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Bioreactor Systems for Tissue Engineering II

Strategies for the Expansion and Directed Differentiation of Stem Cells

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Preface

First of all, the editors of this special volume would like to thank all the authors for their excellent contributions. We would also like to thank Prof. Dr. Thomas Scheper as well as Dr. Marion Hertel and Ingrid Samide from Springer for providing the opportunity to compose this volume and Springer for organizational and technical support.

Tissue engineering represents one of the major emerging fields in modern biotechnology. Tissue engineering combines different disciplines ranging from biological and material sciences to engineering and clinical disciplines. The aim of tissue engineering is the development of therapeutic approaches to substitute diseased organs or tissues or improve their function. Stem cells are early progenitors that may substitute diseased tissues or provide cues for endogenous healing.

Stem cells are present in virtually all tissues. The first chapters describe different sources of stem cells including isolation and expansion. The use of fetal tissues and umbilical cord is discussed as they come from immunoprivileged sites and are considered to be early stem cells. The use of adipose-derived stem cells is discussed as a readily available autologous source. Subsequently, newer techniques for "manufacturing" stem cells from somatic cells using "induced pluripotent stem cell" technology are discussed and described in two chapters. The following chapter deals with bioreactor cultivation of stem cells. Specific tissues such as cartilage and endothelial precursors built the bridge to the last chapters. In those chapters, clinical applications are the focus of interest. It covers a wide range of clinical applications from veterinary orthopedics and human bone diseases until cardiologic applications.

This small overview indicates that we have tried to cover the area of stem cells from isolation, expansion up to clinical applications. The road has been walked already for a substantial distance. However, we are still at the beginning of this exciting new technology.

We hope that this state-of-the-art book is helpful to your research. Please enjoy reading it, as much as we enjoyed preparing it.

Summer 2010

Cornelia Kasper
Ralf Pörtner
Martijn van Griensven

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Alternative Sources of Adult Stem Cells: Human Amniotic Membrane

Susanne Wolbank, Martijn van Griensven, Regina Grillari-Voglauer, and Anja Peterbauer-Scherb

Abstract Human amniotic membrane is a highly promising cell source for tissue engineering. The cells thereof, human amniotic epithelial cells (hAEC) and human amniotic mesenchymal stromal cells (hAMSC), may be immunoprivileged, they represent an early developmental status, and their application is ethically uncontroversial. Cell banking strategies may use freshly isolated cells or involve *in vitro* expansion to increase cell numbers. Therefore, we have thoroughly characterized the effect of *in vitro* cultivation on both phenotype and differentiation potential of hAEC. Moreover, we present different strategies to improve expansion including replacement of animal-derived supplements by human platelet products or the introduction of the catalytic subunit of human telomerase to extend the *in vitro* lifespan of amniotic cells. Characterization of the resulting cultures includes phenotype, growth characteristics, and differentiation potential, as well as immunogenic and immunomodulatory properties.

Keywords Adipogenesis, Expansion, hTERT, Human amniotic cells, Immortalization, Immunomodulation, Immunophenotype, Mesenchymal markers, Osteogenesis, Platelet lysate, Stem cell markers, Telomerase

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Abbreviations

7-AAD	7-Amino-actinomycin D
ALPL	Alkaline phosphatase gene
AP	Alkaline phosphatase
AR	Alizarin red
ASC	Adipose-derived stem cells
BGLAP	Bone gamma-carboxyglutamate protein
BMPR1B	Bone morphogenetic protein receptor 1B
BMPR2	Bone morphogenetic protein receptor 2
BMSC	Bone marrow mesenchymal stem cells
BrdU	5-Bromo-2-deoxy uridine
CBFA1	Core binding factor alpha
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent asssay
FCS	Fetal calf serum
FOI	Fold of induction
hAEC	Human amniotic epithelial cells
hAMSC	Human amniotic mesenchymal stromal cells

HPRT	Hypoxanthine-guanine phosphoribosyltransferase
hTERT	Human telomerase reverse transcriptase
Lep	Leptin
MLR	Mixed lymphocyte reaction
MSC	Mesenchymal stem cells
O-Kit	Mesenchymal stem cell osteogenic stimulatory kit
OO	Oil red O
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Population doubling
PDpT	Population doubling post transduction
PHA	Phytohemagglutinin
PL	Platelet lysate
PPARγ	Peroxisome proliferator-activated receptor gamma
PRP	Platelet-rich plasma
pT	Post transduction
RT-PCR	Reverse transcriptase polymerase chain reaction
SA-β-gal	Senescence associated β-galactosidase
SC	Stem cells
TA	Telomerase activity
TGF-β	Transforming growth factor beta
TRAP	Telomeric repeat amplification protocol
vK	von Kossa

1 Introduction

Various cell sources have been proposed for regenerative medicine, each having their advantages and drawbacks. Since mature cell types are rarely available in sufficient quality and amounts, research has focused on undifferentiated stem cells. Embryonic stem cells are characterized by pluripotency and an unlimited self-renewal capacity [1]. The major drawback of these cells is their high tumorigenic potential. Additionally, their generation is associated with major ethical concerns. In contrast, recovery of adult stem cells is not ethically restricted, tumorigenic conversion was observed only in sparse cases [2], and autologous application is possible. In 1999, Pittenger found that bone marrow not only contained hematopoietic stem cells but also mesenchymal stem cells (MSC) [3]. However, important limitations of bone marrow mesenchymal stem cells (BMSC) are their limited proliferation capacities, their low frequency, and donor site morbidity. Furthermore, decreased differentiation potential with donor age has been reported [4]. During recent years, human adult MSC from various sources including adipose tissue, muscle, connective tissue, skin, placenta, blood, cord blood, synovium,

periosteum, and perichondrium have been established as promising tools in regenerative medicine [5–11]. The first successful cell based therapies for diseases such as myocardial infarction, multiple sclerosis, amyotrophic lateral sclerosis, graft-versus-host-disease, osteogenesis imperfecta, and Crohn's fistula have been conducted [12–17].

1.1 Stem Cell Characteristics of Amnion-Derived Cells

Placenta derived cells, in particular those from amniotic membrane, have been described to combine qualities from both embryonic and adult stem cells, with a differentiation capacity to derivatives of all three germ layers, and a lack of tumorigenicity [18, 19]. Amniotic membrane is the innermost of the fetal membranes and consists of a single layer of epithelial cells residing on a basement membrane, overlying a stromal layer. Human amniotic epithelial cells (hAEC) and human amniotic mesenchymal stromal cells (hAMSC), respectively, can be released separately from these two layers by differential enzymatic digestion [19–21]. Both of these cell types have been described to express markers of mesenchymal and embryonic stem cells [18, 19, 22–25]. What makes these cells especially attractive is that large amounts can be isolated from an uncontroversial material that is usually discarded after birth. Most importantly, immunosuppressive characteristics of amniotic cells might render allogeneic application possible [19, 24, 26]. Furthermore, their fetal origin may provide amniotic cells not only with stem cell potential but also with an immunoprivileged status [27]. Human amnion is widely used in surgery and wound treatment for burned skin, decubitus ulcers, and in ophthalmology [28, 29]. When transplanting amniotic membrane intracorneally or under the kidney capsule, no rejection but only a mild cell-mediated reaction was observed [27].

All these characteristics would make amniotic cells ideal candidates for tissue engineering and their application in regenerative medicine. For this purpose, cells can theoretically be used directly after isolation, or after *in vitro* cultivation, the latter of which permits a gain in cell numbers, but important disadvantages are increases in the risk of contamination with pathogens, accumulation of mutations, and loss of differentiation potential and functionality.

1.2 Expansion and Cryoconservation of Amnion-Derived Cell: Towards Cell Banking

To clarify the effect of *in vitro* culture on the quality of amnion-derived cells, a thorough characterization comparing these cells before and after cultivation has been performed.

Applicability of these cells for allogeneic transplantation and stem cell based therapies could further be boosted by standardized collection, quality control, and careful selection of functional and safe cell banking products. However, in order to provide sufficient stem cell numbers for cell banking and cell based therapies, their limited replicative potential has to be overcome. Regarding this aim, we followed two strategies: (1) optimization of the expansion medium using human derived growth supplements instead of fetal calf serum (FCS) and (2) introduction of the catalytic subunit of human telomerase.

1.2.1 Effect of In Vitro Expansion on Amnion-Derived Stem Cells

As a consequence of the adaptation processes to the artificial cell culture environment and/or the possible enrichment of clones that have a growth advantage *in vitro*, the phenotype of cells may change during cultivation. Such alterations during *in vitro* cultivation have been described for BMSC [3] and in detail for adipose-derived stem cells (ASC) [30–32].

We systematically analyzed the surface antigen expression profile of hAEC directly after isolation and in the course of *in vitro* cultivation, with a focus on mesenchymal and embryonic stem cell markers and investigated possible functional consequences of *in vitro* cultivation regarding their osteogenic and adipogenic differentiation potential.

1.2.2 Strategies to Circumvent Growth Limitation In Vitro: Use of Platelet Lysate During Expansion and Cryopreservation

Culturing mammalian cells usually involves expansion in cell culture medium supplemented with FCS. Furthermore, FCS is also a crucial component of cryopreservation media. While FCS is the golden standard to supplement research cell culture media, its application for cell based therapies should be minimized as it bares the risk for transmission of pathogens including prions, viruses and zoonoses [33]. Immunological *in vitro* reactions to FCS after cultivation have already been demonstrated [34, 35]. In addition to the reported disadvantages of FCS there is a predicted shortage in FCS in the next few years resulting in 50% increased purchase prices [36].

Hence, well-screened human sources for growth factors would be favorable for cell therapy. As such, platelets may offer a viable alternative to FCS. They contain in their α-granules growth factors including platelet derived growth factor, basic fibroblast growth factor, insulin-like growth factor, and transforming growth factor beta (TGF-β) [33] whereby TGF-β1 is the most abundant [37]. These growth factors play important roles during wound healing by exerting above all mitogenic activity.

Platelet derived products – such as platelet lysate (PL) or platelet-rich plasma (PRP) – have already been proposed as culture supplement for several cell types

including BMSC [33, 37–47], umbilical cord blood MSC [48], ASC [43, 49], and stromal cells from dental pulp and trabecular bone [43] showing increased clonogenic efficiency and proliferative capacity compared to standard FCS culture. PL also stimulates proliferation and collagen production of human tenocytes and increases the gene expression of matrix-degrading enzymes and angiogenic growth factors [50]. Furthermore, myelomas, hybridomas, hepatocytes, fibroblasts, and epithelial cells have already been evaluated using PL with regard to cell growth, viability, and production efficiency [51]. Growth stimulation upon PL treatment was also demonstrated for primary chondrocytes. However, PL failed to support a chondrogenic phenotype [52–54] in contrast to BMSC cultures showing increased chondrogenic marker genes in presence of PRP [55]. Primary human skeletal muscle cells showed decreased differentiation capacity into myotubes and impaired functionality [56]. Proliferation of primary human osteoblasts was not affected by addition of PRP to the culture medium [57]. For human dermal and gingival fibroblasts, contradicting results were obtained for platelet derived products as culture supplements ranging from growth suppression [58] to growth promotion [59, 60]. Using ASC, Davenport et al. showed that PL only initially supported cell proliferation but led to growth arrest shortly after first subcultivation [61]. In contrast, addition of thrombin activated PRP to the culture medium increased ASC proliferation and retained their differentiation capacity during long-term culture [49].

Moreover, platelet derived products and BMSC have already been used clinically both to treat distraction osteogenesis of the lower extremity in patients with achondroplasia and hypochondroplasia yielding accelerated bone regeneration [62] and also to treat successfully a patient with severe radiation burn [63].

However, the influence of platelet derived products for the cultivation of cells isolated from amniotic membrane has not been addressed before.

1.2.3 Strategies to Circumvent Growth Limitation In Vitro: Introduction of hTERT

Stem cells needed at therapeutic doses, especially in adults, may require extensive *in vitro* expansion. In this regard, one major drawback of these cells is their low proliferative capacity and limited *in vitro* life span before reaching an irreversible growth arrest also termed replicative senescence [19]. Additionally, long-term cultures of human MSC may show altered or reduced responsiveness to differentiation signals [64].

One strategy to circumvent these limitations is the introduction of the catalytic subunit of human telomerase reverse transcriptase (hTERT) which has been reported to extend the cellular life span of numerous cell types including normal fibroblasts, endothelial or epithelial cells [65–67], *in vitro* propagated tumor cells [68–70], and also of stem cells [71, 72]. It has been shown that hTERT immortalized human MSC originating from sources such as bone marrow and adipose tissue maintain their differentiation potency [72–74]. We report in this section the

establishment and the characterization of the first hTERT immortalized hAMSC lines including their immunomodulatory functions, a crucial factor for using these cell lines in allogeneic cell therapies [75]. Therefore, if cell banking is intended, it is important to monitor the cells' ability to alloactivate peripheral blood mononuclear cells (PBMC) as well as to modulate the proliferation of activated PBMC.

2 Stem Cell Characteristics and Immunomodulatory Potential of Human Amnion-Derived Stem Cells

2.1 Isolation of Separate Populations of hAEC and hAMSC

Placentae were collected from Cesarean sections after obtaining informed consent of the mothers according to the approval of the local ethical committee. Amnion was peeled off the placenta, washed extensively with phosphate buffered saline (PBS) at 4°C, and dissected in 2–3 cm^2 pieces. Half of these were digested for 3×20 min with 0.05% trypsin/EDTA (PAA, Austria), the other half for 2 h with 1 mg/mL collagenase I (Biochrom, Austria) for isolation of hAEC and hAMSC, respectively. Hematoxylin/eosin staining of paraffin embedded sections of amnion demonstrates that digestion of amniotic membrane with trypsin and collagenase left an essentially intact mesenchymal and epithelial layer (Fig. 1a).

After addition of ice-cold PBS, cell suspensions were filtered through a 100-μm cell strainer, centrifuged, and seeded in culture flasks at a density of 7×10^3 cells/cm^2 for hAMSC and 14×10^3–21×10^3 cells/cm^2 for hAEC in EGM-2

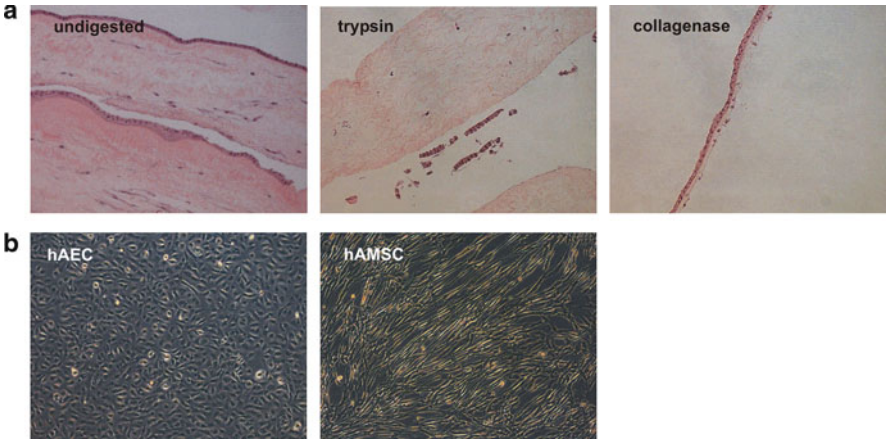

Fig. 1 Isolation of pure fractions of hAEC and hAMSC. (**a**) Hematoxylin and eosin stain of fresh amnion (undigested) and after digestion with trypsin and collagenase, as performed for isolation of hAEC and hAMSC, respectively. (**b**) Epithelial and mesenchymal morphology of hAEC and hAMSC, respectively

(Lonza, Belgium). The resulting cultures are composed of pure populations with a clearly distinguishable epithelial and mesenchymal morphology, respectively (Fig. 1b).

2.2 Stem Cell Characteristics and Immunomodulation of hAEC and hAMSC

Both amniotic cell populations are routinely characterized by a common surface marker expression profile including the presence of CD73, CD90, CD105, and MHC I, and the concomitant absence or low levels of CD34, CD45, and MHC II, analyzed by flow cytometry. Purity of amniotic subpopulations could be determined by CD49d (α4-integrin) expression which was $2 \pm 2.4\%$ in the hAEC and $96 \pm 3.9\%$ in the hAMSC population.

To evaluate the reproducibility of differentiation of hAEC and hAMSC, osteogenic differentiation was induced 24 h after seeding by changing the medium to Mesenchymal Stem Cell Osteogenic Stimulatory Kit (O-Kit, Stemcell Technologies, Canada) and maintaining these cultures for 21 days. Adipogenic differentiation was performed according to Portmann-Lanz et al. [23]. Osteogenic differentiation was demonstrated by spectrophotometric assessment of Alizarin red (AR). Typically, osteogenic differentiation of both cell types, hAEC (at P1 or P2) and hAMSC (at P2), was successfully induced in three of four cases. Adipogenic differentiation was evident for two of four hAMSC isolations, while, in contrast to published data [22, 23], hAEC did not differentiate along the adipogenic lineage in our hands (data not shown).

For investigating immunomodulation *in vitro*, amnion-derived cells were cocultured with PBMC, isolated from whole blood as in mixed lymphocyte reactions (MLRs). For this, 5×10^4 cells of two different allogeneic PBMC populations were cocultured in 100 μL PBMC medium/well (RPMI1640, 9% FCS, 2 mM L-glutamine, 100 U/mL penicillin G, and 0.1 mg/mL streptomycin) in triplicates in 96-well flat bottom plates. Amnion-derived cells were seeded in the wells and allowed to adhere before adding PBMC. The stem cells (SC) were added at SC/PBMC ratios of 1:1 (5×10^4 SC), 1:2, 1:4, 1:8, and 1:16. On day 5, 10 μM 5-bromo-2-deoxyuridine (BrdU) was added and BrdU ELISA (Roche) was performed on day 6 according to the manufacturer's instructions. Similarly, for phytohemagglutinin (PHA) activation assay, 5×10^4 PBMC were activated by 5 μg/mL PHA (Sigma) on day 3 of the culture. To examine interaction between allogeneic SC and unstimulated PBMC, SC were cocultured with unstimulated PBMC at 1:1 (5×10^4 SC), 1:2, 1:4, 1:8, and 1:16 ratios in 100 μL. On day 4, 10 μM BrdU was added. The inhibitory effect of SC was calculated as PBMC proliferation (%) = $(E_{STIM+SC} \div E_{STIM}) \times 100$. $E_{STIM+SC}$ = mean absorption of stimulated PBMC cocultured with allogeneic SC; E_{STIM} = mean absorption of stimulated PBMC. Data were analyzed by one-way ANOVA and Tukey's multiple

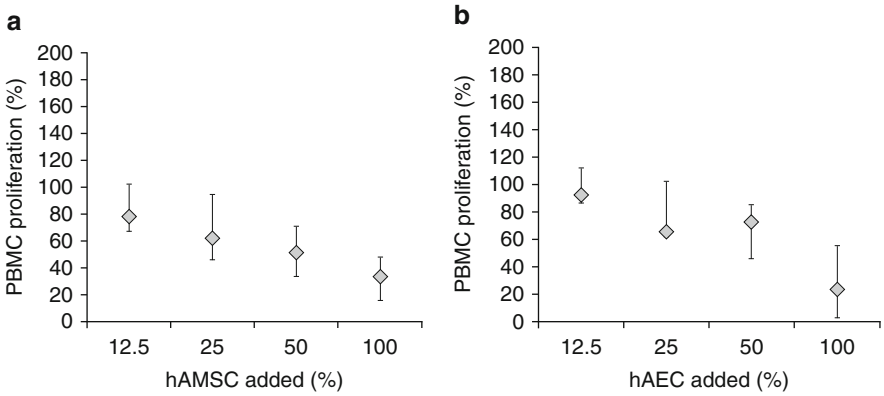

Fig. 2 hAMSC and hAEC inhibit MLR-activated PBMC in a cell dose-dependent manner. PBMC were cocultured with equal amounts of allogeneic PBMC and different amounts of third party (**a**) hAMSC ($n = 12$), (**b**) hAEC ($n = 9$). Median ± Q1 and Q3 are depicted

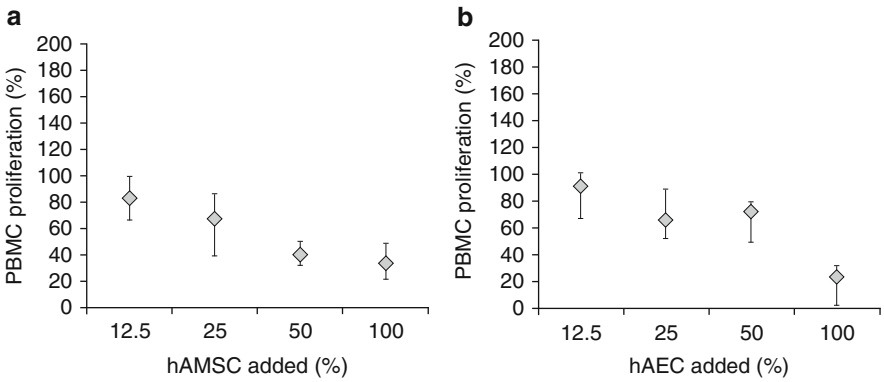

Fig. 3 hAMSC and hAEC inhibit PHA-activated lymphocyte proliferation in a cell dose-dependent manner. PBMC were cocultured with (**a**) hAMSC ($n = 12$), (**b**) hAEC ($n = 8$). Median ± Q1 and Q3 are depicted

comparison test. Data sets of cells at low vs high population doublings (PDs) were compared by two-tailed Student's *t*-test. A *p*-value less than 0.05 was considered as significant.

hAMSC and hAEC inhibited proliferation of activated PBMC in a dose-dependent manner as demonstrated by a decrease in proliferation with increasing stem cell amounts. SC were most effective when added in equal cell numbers compared to PBMC, significantly reducing PBMC proliferation in MLR experiments to a level of 34% (range 3–73%) in the case of hAMSC (Fig. 2a) and 23% (range 0–72%) in the case of hAEC (Fig. 2b). When PBMC were activated by PHA, similar inhibition was reached, in detail 33% (range 12–66%) for hAMSC (Fig. 3a), and 28% (range 0–60%) for hAEC (Fig. 3b). The lowest SC dose resulting in significant inhibition of lymphocyte response was 25%, in single cases even 12.5%.

3 Phenotypic Shift and Reduced Osteogenesis During In Vitro Expansion of Human Amnion Epithelial Cells

For tissue engineering purposes, cells may be applied either directly after isolation from the tissue or after a period of *in vitro* expansion to obtain higher cell numbers. In order to investigate the advantages and drawbacks of these strategies we compared freshly isolated and cultivated hAEC regarding their surface antigen expression profile and their osteogenic differentiation capacity.

3.1 Shift in Surface Antigen Expression During Cultivation of hAEC

To investigate the impact of *in vitro* expansion on the immunophenotype of cells with potential for regenerative medicine, we carefully characterized the surface antigen profile of hAEC directly after isolation and during cultivation by flow cytometry. We focused on hAEC, as recovery of primary hAMSC is usually too low for thorough analysis. For this purpose, freshly isolated cells and cells during culture were immunostained for CD14, CD34, CD45, CD13, CD29, CD44, CD49c, CD49d, CD49e, CD54, CD73, CD90, CD166, Ki67 (BD, Austria), CD105 (Abcam) and SSEA-4, TRA-1-60, TRA-1-81 (Chemicon), by 7-AAD (BD) for dead cells and measured by flow cytometry.

Surface antigens were clustered into four groups, according to their expression patterns (representative histograms are depicted in Fig. 4a, summarized in Fig. 4b). The first group comprises CD49d (integrin α4; used to differentiate hAEC from hAMSC) and the hematopoietic markers CD14, CD34, and CD45. These antigens are hardly detectable on freshly isolated hAEC (preP0) and remain at similar levels during passaging.

The surface antigens of the second group are uniformly expressed at high levels, both in primary isolates (preP0) and after further cultivation. This group comprises the stromal cell markers CD29 (integrin β1), CD49c (integrin α3), CD73 (ecto-5′-nucleotidase), and CD166 (ALCAM), and the embryonic stem cell marker SSEA-4.

Group 3 consists of the stromal cell associated markers CD13 (aminopeptidase N), CD44 (HCAM), CD49e (integrin α5), CD54 (ICAM-1), CD90 (Thy-1), and CD105 (endoglin), which are low (medium to undetectable) directly after isolation (preP0) and are rapidly increased during *in vitro* cultivation.

Two additional embryonic stem cells markers, TRA-1-60 and TRA-1-81 (group 4), are characterized by medium expression in preP0 cells, which decreases upon cultivation.

As in vivo, amniotic cells reside within a tissue that remains of approximately the same size during the last weeks of pregnancy, these cells would probably be in a quiescent state directly after isolation, but start dividing upon transfer into tissue culture medium. Therefore, we tested the hypothesis that upregulation of group 3 antigens might be associated with re-entry of the cells into the cell cycle. Only

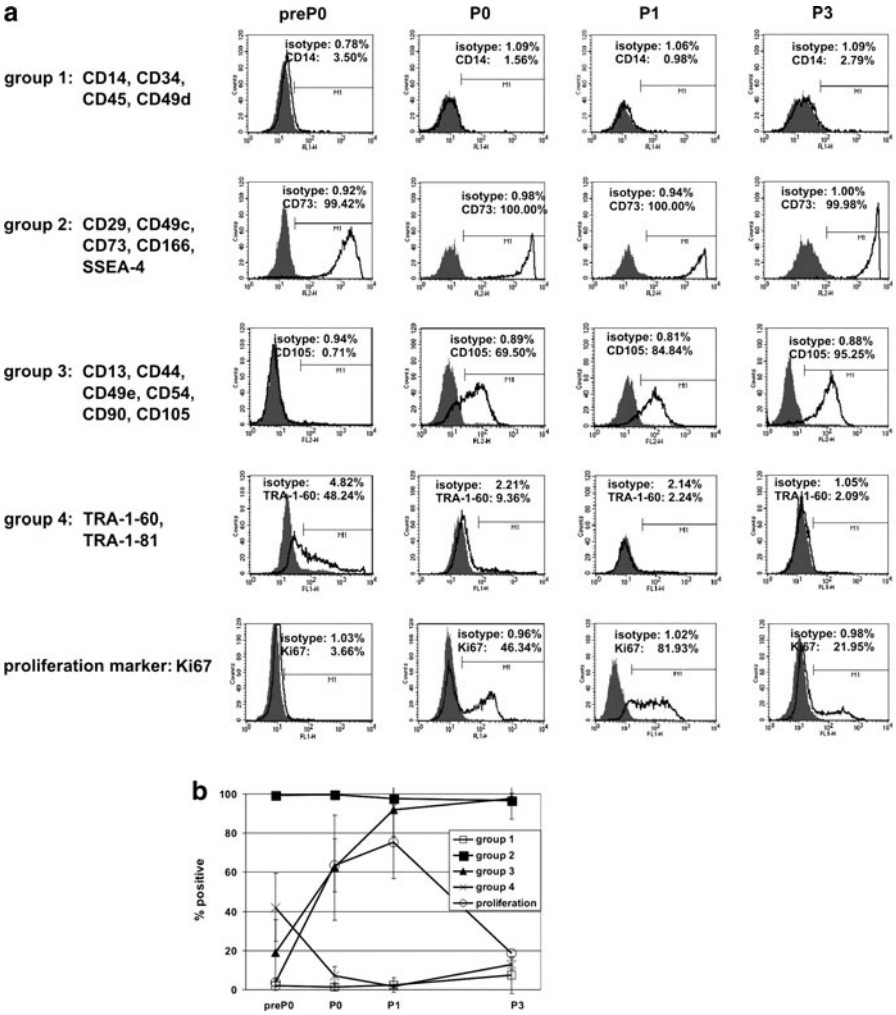

Fig. 4 Surface antigen expression of four freshly isolated (prePO) hAEC strains and during cultivation at various passages (P0–P3) by flow cytometry. (**a**) Antigens were grouped according to their expression profile during cultivation (*see text*) and representative histograms of one member of each group are shown. *Gray peaks* represent unspecific isotype controls, *solid lines* represent the specific antibodies. (**b**) Summary: shown are means and corresponding standard deviations, calculated from the data of all antigens of each group of all four hAEC isolations (hAEC 87, −88, −90, and −91) at the indicated passages

2–6% of freshly isolated hAEC (prePO) were stained for the proliferation marker Ki67, which is expressed in all phases of the cell cycle but not in G0. After a few days in culture, Ki67 expression increased dramatically (Fig. 4a, b), concomitant with the observed upregulation of group 3 antigens. However, expression of group 3 antigens is not dependent on proliferation of hAEC, as expression remained high

when proliferation slowed down after several passages *in vitro*, concomitant with a drastic decrease in Ki67 staining (Fig. 4a, b).

3.2 Osteogenic Differentiation Potential of hAEC Decreases upon In Vitro Cultivation

We addressed the question as to whether the observed shift in mesenchymal and embryonic stem cell markers during cultivation of hAEC (Fig. 4) is associated with alterations of their functional phenotype, i.e., their capacity to differentiate along the adipogenic and osteogenic lineages. Therefore, adipogenic and osteogenic conditions (as described in Sect. 2.1) were applied to different hAEC isolations seeded directly after isolation (P0) and after cultivation (P2 or P3). In addition to mineralization, quantitative real time PCR was performed analyzing expression of RUNX2/CBFA1 (core binding factor alpha), alkaline phosphatase (ALPL), bone gamma-carboxyglutamate protein (BGLAP, osteocalcin), bone morphogenetic protein receptor 1B (BMPR1B), and bone morphogenetic protein receptor 2 (BMPR2) using a light cycler TM480 (Roche) and Taqman gene expression assays (Applied Biosystems). Expression values were normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Similar to passaged hAEC, no adipogenic differentiation was observed using four strains of freshly isolated hAEC (data not shown). In contrast, the same strains showed predominantly stronger mineralization ability at P0 (in three out of four cases) when compared to passaged cells (P2–P3). Interestingly, preliminary results with hAMSC suggest similar mineralization and lipid accumulation after induction of P0 vs P2 cells (data not shown). We confirmed mineralization by analysis of mRNA levels of selected genes involved in osteogenesis. In freshly isolated hAEC (P0) all osteogenic markers were upregulated upon cultivation in O-Kit for 14 and 21 days (Fig. 5). In cultivated hAEC (P1), RUNX2 and ALPL were also increased under osteogenic conditions whereas virtually no alteration in transcription of BGLAP, BMPR1B, and BMPR2 was observed.

4 Platelet Lysate for FCS-Free Expansion and Cryoconservation of Amnion-Derived Cells

For producing PL, platelet concentrates from 36 healthy donors that could no longer be used for patients were pooled, frozen at −80°C, and thawed quickly in a water bath at 37°C, resulting in growth factor release from bursting platelets. Platelet debris was removed by centrifugation at 2,000×g for 10 min while the PL was filtered using a 0.22-μm filter, aliquoted, and stored until application at −80°C.

For determining growth kinetics, cells were isolated from three donors as described in Sect. 2.1, and 2.5×10^5 cells were seeded in T-25 flasks and cultured

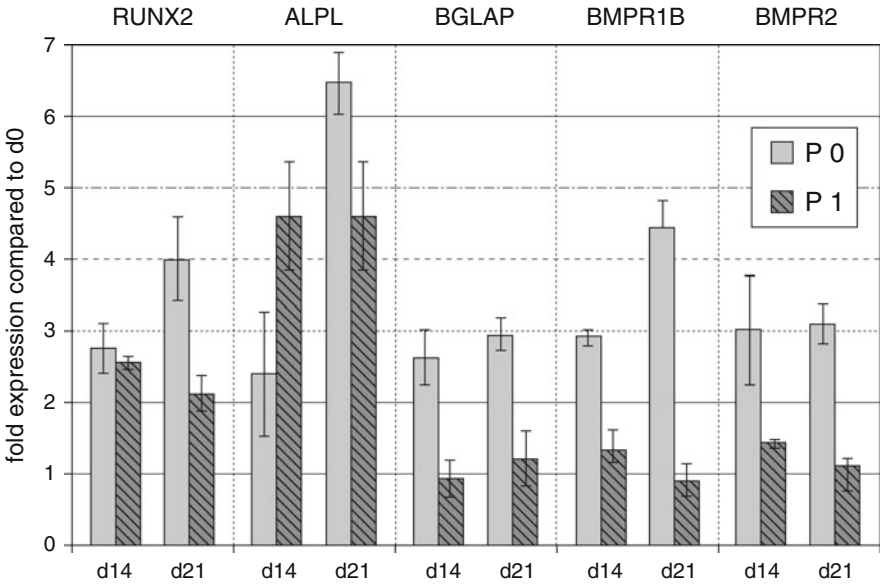

Fig. 5 Osteogenic differentiation of hAEC isolations seeded directly after isolation (P0) and after cultivation (P1). Expression levels of selected genes implicated in osteogenesis, determined using quantitative real-time PCR. Expression levels after cultivation in O-Kit for 14 and 21 days (d14 and d21) were normalized to the levels before induction (d0). Shown are means and standard deviations of three measurements from two individual donors

for about 100 days in PL expansion medium (DMEM-LG & Ham's F12, 5% PL, 2 mM L-glutamine, 100 U/mL Penicillin, 0.1 mg/mL Streptomycin (PAA), 2 U/mL Heparin (Biochrom)) at 37°C, 5% CO_2, and 95% humidity. PD was calculated at each subcultivation using the formula: ΣLN (cells harvested/cells seeded)/LN(2).

hAMSC from all three donors cultured in PL showed between 21 and 32 PD without growth arrest whereas control hAMSC cultured in EGM-2 (Lonza), a commercially available medium containing 2% FCS, showed only 5 PD before proliferation totally ceased (Fig. 6a).

Not only during expansion but also during cryopreservation, substitution of FCS would be favorable for establishing cell banks. In preliminary experiments, several media containing PL (5%, 90% PL) were compared to standard FCS media (10%, 90% FCS) as well as a serum-free medium (CryoSFM, PromoCell) for cryopreservation of hAMSC. Then 1×10^6 P1 cells were resuspended in the respective medium and frozen at a freezing rate of −1°C/min. From these preliminary data, the best conditions, namely 5% PL medium (5% PL, 10% dimethylsulfoxide (DMSO), 85% DMEM-LG), 90% FCS medium (90% FCS, 10% DMSO), and CryoSFM were chosen for further investigation.

After thawing, cell viability was assessed by trypanblue exclusion assay. Additionally, growth kinetic studies were performed to evaluate characteristics of hAMSC before and after cryopreservation. Cells cryopreserved in PL showed

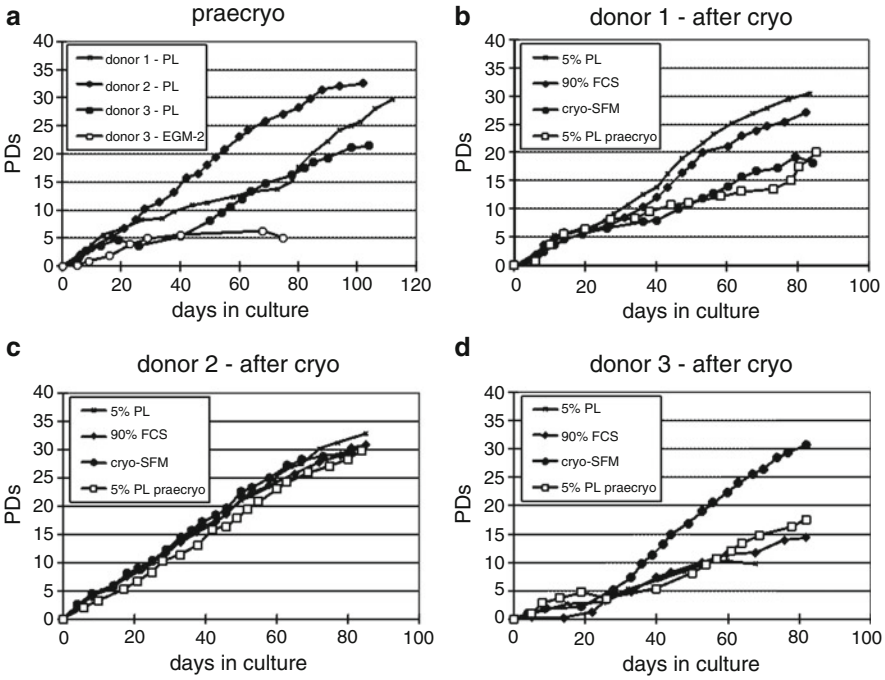

Fig. 6 Growth characteristics of hAMSC from three donors before (**a**) and after cryopreservation (**b–d**) cultivated in medium supplemented with either 5% PL or EGM-2 and cryopreserved in FCS, PL containing media or serum-free Cryomedium (CryoSFM). *PDs* cumulative population doublings

lower cell viability after thawing when compared to those stored in FCS medium or serum-free CryoSFM (Fig. 7). While growth kinetics after thawing seemed to be unaffected by the cryopreservation medium applied, strong impact of the donors on PD was evident (Fig. 6b–d). An observed phenomenon was the low attachment capacity of hAMSC cryopreserved in 5% PL. Hence, coating experiments with gelatine (1% in PBS) or "Coating matrix Kit" (Invitrogen) were performed but attachment of cells to the culture vessel surface could not be substantially improved (data not shown).

5 hTERT Induced Extension of In Vitro Life Span of Amnion-Derived Stem Cells

For introduction of hTERT a retroviral transfection system was chosen. Therefore, the cDNA of hTERT (kindly provided by Geron Corp.) was inserted into the retroviral vector pLXSN (Clontech Laboratories Inc.) and retroviral particles

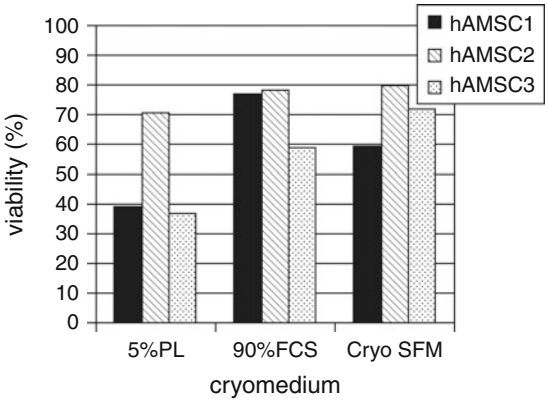

Fig. 7 Cell viability of hAMSC from three donors after cryopreservation in PL, FCS containing media or serum-free Cryomedium (CryoSFM) and thawing as determined by trypanblue exclusion assay. Data are presented in % of viability at freezing

were generated as described previously [70]. Gene transfer was performed at early PD (<PD8) according to the manufacturer's instructions (Clontech Laboratories Inc.). Then 24 h post transduction transfectants were selected using 200 μg/mL Geneticin Sulfate G418 and arising cell clones were grown as mass culture. PD of transduced cell lines were calculated starting with the first passage post transduction (PDpT) using the formula stated in Sect. 4. Telomerase activity (TA) was determined using a modification of the real-time telomeric repeat amplification protocol (TRAP) assay as described in detail previously [70] and calculated relative to that of HEK293 cells (positive control). For determination of senescence, associated β-galactosidase (SA-β-gal) activity cells were fixed with 3% formaldehyde and stained as described in detail previously [76]. For characterizing phenotype, differentiation potential, and immunomodulatory properties, protocols according to Sect. 2.2 were performed. For quantitative evaluation, AR was measured after extraction using 20% methanol/10% acetic acid at 450 nm. For quantification of intracellular alkaline phosphatase (AP) activity, washed cells were frozen and thereafter incubated in 0.5% Triton X-100. After incubation with 4-nitrophenolphosphate, samples were measured at 405/620 nm. In addition to osteogenic marker genes peroxisome proliferator-activated receptor gamma (PPARγ) and leptin (Lep) were evaluated as adipogenic marker genes by quantitative RT-PCR as described in Sect. 3.2.

Immortalized stem cell lines (originating from the mesenchymal layer of the two amniotic membrane donors hAMSC76 and hAMSC83) were established by overexpression of hTERT. Human stem cells were isolated from amnion and propagated *in vitro* until they reached replicative senescence. Representative growth curves of hAMSC76 are shown in Fig. 8a. Senescence was evidenced by growth arrest, large and flat cell morphology (Fig. 8b), and SA-β-gal activity (Fig. 8c). Upon ectopic expression of hTERT stem cell populations were immortalized (so far expanded to at least PD60 with no signs of growth retardation; Fig. 9a). Furthermore, hTERT overexpression maintained many characteristics of the original cellular phenotype. Figure 9b demonstrates fibroblastoid morphology of transduced cells (hAMSC76telo-PD78pT, hAMSC83telo-PD43pT) comparable to early passage

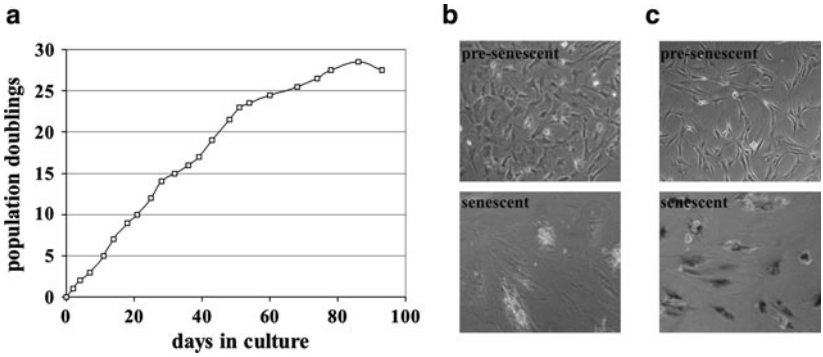

Fig. 8 Growth characteristics and morphology of hAMSC. (**a**) Cells were grown in vitro until replicative senescence. Representative growth curve of hAMSC76 is shown. (**b**) Phase contrast microscopy and (**c**) staining for SA-β-galactosidase activity of early and late passage cells. Magnification in b, c: ×100

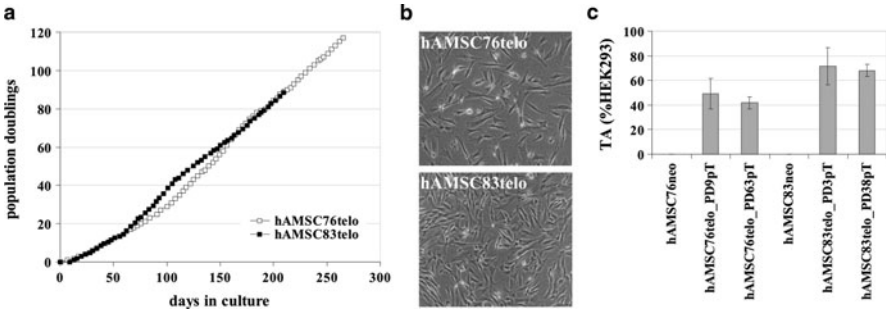

Fig. 9 Growth potential and morphological characteristics of hTERT-transduced hAMSC. (**a**) Growth curves of hTERT-transduced cell lines. (**b**) Phase contrast microscopy of hTERT-transduced immortalized cell lines. (**c**) TRAP assays at two different PDs post transduction (PDpT) demonstrate telomerase activity in cell lines. *neo* Vector control, *telo* hTERT-transduced; in b: ×100

parental counterparts. Telomerase activity after transduction was verified by TRAP assay (Fig. 9c). In contrast to empty vector control cells (hAMSCneo), hTERT-transduced cell lines expressed significant telomerase activity at early as well as higher PDpT (at least PD38pT) when compared to HEK293 control cells (49–72% of HEK293).

Expression of selected hematopoietic (CD14, CD34, and CD45 negative) and mesenchymal markers (CD73, CD90, and CD105 positive) on hTERT-transduced cell lines were similar to their parental cultures, also after prolonged *in vitro* propagation (hAMSC76telo-PD84pT, hAMSC83telo-55pT; Table 1). Interestingly, the major population of hTERT-transduced hAMSC lost expression of the mesenchymal marker CD90. Therefore, hAMSC83telo were characterized in more detail, i.e., at several PD and for additional antigens (Table 2). At early PD after

Table 1 Surface antigen expression of nontransduced (normal) and hTERT-transduced (telo) hAMSC. hAMSC76 and hAMSC83 were analyzed at PD6 and 5, their hTERT-transduced counterparts at population doubling 84 and 55 post transduction, respectively

Antigen	hAMSC76		hAMSC83	
	Normal	Telo	Normal	Telo
CD14	0.5	7.0	2.6	10.5
CD34	0.0	0.1	0.1	0.1
CD45	0.0	11.6	0.0	2.0
CD73	96.0	100.0	99.9	100.0
CD90	100.0	53.3	100.0	8.7
CD105	95.7	99.8	92.5	99.9
HLA ABC	98.9	99.8	99.4	99.7
HLA DR	0.5	2.6	0.2	2.3

Table 2 Detailed flow cytometric characterization of hTERT-transduced hAMSC83telo during prolonged in vitro cultivation, compared to the nontransduced counterpart. Additional antibodies were purchased from BD (CD13, CD29, CD44, CD49c CD49d CD49e, CD54, CD166), Chemicon (SSEA-4, TRA-1-60, TRA-1-81 and Oct-4), Santa Cruz Biotechnology (Vimentin)

Antigen	Normal	Telo		
	PD3	PD26pT	PD55pT	PD96pT
CD13	100.0	100.0	99.7	99.8
CD14	5.1	2.1	10.5	6.4
CD29	99.9	n.d.	99.6	99.8
CD34	0.4	0.1	0.1	0.2
CD44	100.0	n.d.	99.4	99.9
CD45	2.7	0.1	2.0	4.8
CD49c	100.0	n.d.	99.8	99.8
CD49d	98.2	n.d.	99.7	99.9
CD49e	100.0	n.d.	99.7	99.9
CD54	72.0	n.d.	89.0	98.7
CD73	100.0	76.6	100.0	100.0
CD90	100.0	100.0	8.7	22.8
CD105	98.5	95.8	99.9	99.8
CD166	100.0	n.d.	99.8	99.8
HLA ABC	100.0	100.0	99.7	99.9
HLA DR	7.2	0.7	2.3	5.2
SSEA-4	26.6	81.7	99.6	85.6
TRA-1-60	6.4	n.d.	3.4	0.9
TRA-1-81	6.1	n.d.	6.4	0.5
Oct-4	62.1	n.d.	72.9	89.2
Vimentin	100.0	n.d.	100.0	n.d.

n.d. Not determined; *PD* population doubling; *pT* post transduction

transduction, hAMSC83telo were still homogenously positive for CD90; however at PD55pT, only 8.7% of the cells expressed this marker. This subpopulation remained detectable after a further 41 PDs (PD96pT). With the exception of SSEA-4, the expression of which increased after hTERT transduction, all other

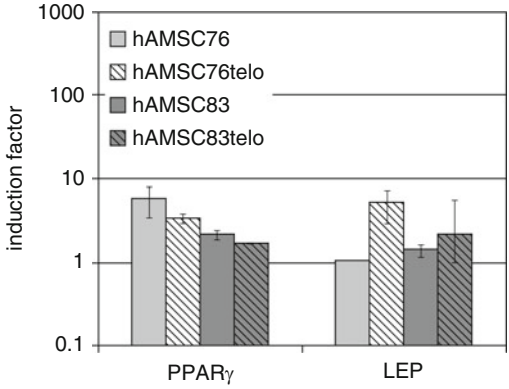

Fig. 10 Adipogenic differentiation potential of nontransduced and hTERT-transduced hAMSC 3 weeks after induction. Relative expression of peroxisome proliferator-activated receptor gamma (PPARγ) and leptin 2 weeks after adipogenic induction of nontransduced and hTERT-transduced hAMSC. Expression levels are normalized to HPRT and presented relative to d0 cultures (set to 1). Means and SDs of two individual experiments are displayed

antigens tested showed no alteration. Analysis of the cellular karyotype revealed that hTERT transduction did not induce abnormalities in chromosomal number or structure since both, nontransduced stem cells and the hTERT cell lines showed a normal karyotype. Additionally, soft agar assays showed no indication for a tumorigenic conversion upon hTERT transduction (data not shown).

After introduction of hTERT, amnion-derived stem cell lines showed a similar differentiation potential towards the adipogenic and osteogenic lineage when compared to the nontransduced counterparts. hAMSC generally show a low differentiation potential towards the adipogenic lineage as demonstrated by OO staining. Although singular hAMSC76telo cells gained the capacity for lipid accumulation, these rare events were not quantifiable (data not shown). On the level of adipogenic marker genes, quantitative real-time PCR revealed low levels of PPARγ expression and induction of leptin transcription in hAMSC (Fig. 10). When testing for osteogenic differentiation, significant mineral deposition of all hAMSC lines was observed, as analyzed by quantification of AR staining (Fig. 11a). Also, a low but significant increase of AP activity (Fig. 11b) as well as induction of mRNA levels of AP at very low levels was seen (Fig. 12a). Osteocalcin mRNA was induced in all hAMSC during differentiation (Fig. 12b). In order to test immunomodulation of the hTERT immortalized stem cell lines, their suppressive effect on MLR- or PHA-activated lymphocyte proliferation was analyzed (Fig. 13). The tested cells inhibited MLR-activated PBMC proliferation in a cell dose-dependent manner. hTERT-transduced hAMSC inhibited significantly at a 1:8 SC/PBMC ratio (Fig. 13a), parental hAMSC even at 1:16. Similarly, when stem cells were cocultured with PHA-activated PBMC, the inhibitory potency of hAMSC was unaltered after hTERT overexpression, inhibiting significantly at a ratio of 1:8 (Fig. 13b).

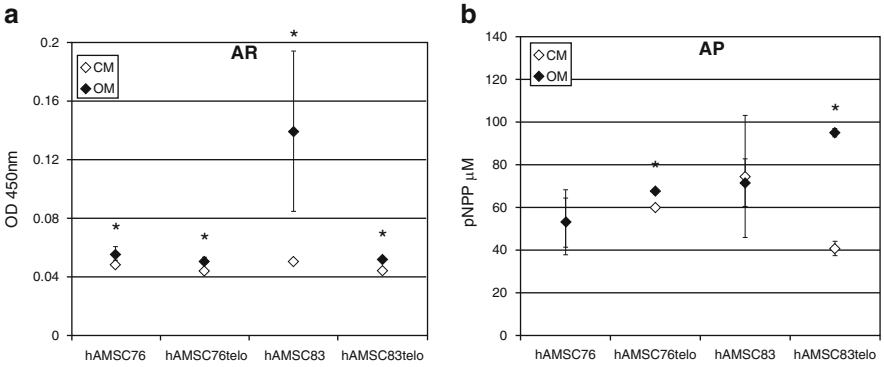

Fig. 11 Osteogenic differentiation potential of nontransduced and hTERT-transduced hAMSC 3 weeks after induction. Osteogenic differentiation demonstrated by Alizarin red (AR) quantification (**a**) and alkaline phosphatase (AP) (**b**) of hAMSC. Differences between control cultures (CM) and osteogenic differentiation cultures (OM) with $p < 0.05$ were regarded as significant

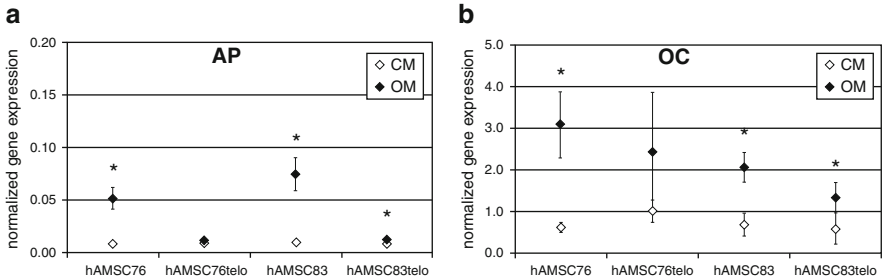

Fig. 12 Expression levels of alkaline phosphatase (AP) (**a**) and osteocalcin (OC) (**b**) 2 weeks after osteogenic induction of nontransduced and hTERT-transduced hAMSC. Expression levels in osteogenic medium (OM) and control medium (CM) are presented normalized to HPRT. Means and SDs of three individual experiments are displayed. Differences between control cultures (CM) and osteogenic differentiation cultures (OM) with $p < 0.05$ were regarded as significant

6 Discussion

6.1 Stem Cell Characteristics and Immunomodulatory Potential of Human Amnion-Derived Stem Cells

Taken together, our data clearly show a cell dose dependency of the immunomodulatory effect of amnion-derived stem cells, which corroborates published results for MSC from adipose tissue or bone marrow [77–81]. Furthermore, we demonstrate cell dose-dependent immunosuppression for the two distinct amniotic stem cell types which were characterized by differential expression of α4-integrin. Interestingly,

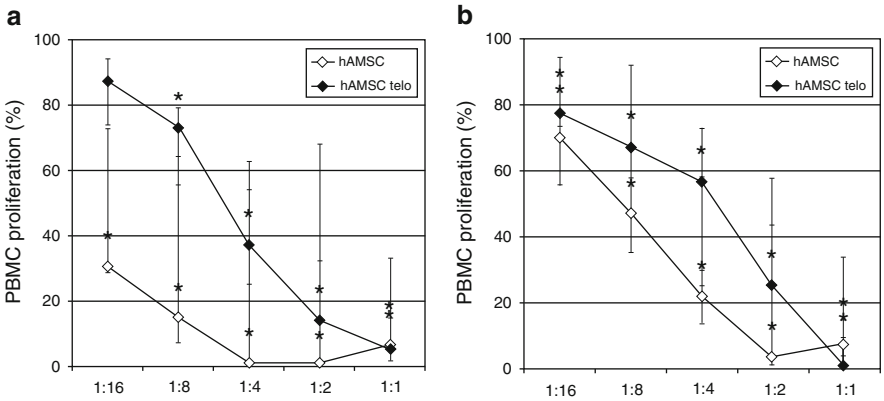

Fig. 13 Immunomodulation of nontransduced and hTERT-transduced hAMSC. (**a**) hAMSC (76 $n = 4$, 83 $n = 3$) inhibit mixed lymphocyte reaction (MLR) in a cell dose-dependent manner. (**b**) hAMSC (76 $n = 4$, 83 $n = 4$) inhibit phytohemagglutinin-activated lymphocyte proliferation in a cell dose-dependent manner. PBMC proliferation is calculated as percentage of uninhibited proliferation. $p < 0.05$ was regarded as significant inhibition. Median ± Q1 and Q3 are depicted. *Asterisk* indicates significant inhibition of proliferation

although epithelial and mesenchymal fractions show distinct morphology and marker expression, they have similar potency to modulate immunoreactions *in vitro*.

6.2 Phenotypic Shift and Reduced Osteogenesis During In Vitro Expansion of Human Amnion Epithelial Cells

We show here that differentiation of amniotic cells strongly depends on the donor. Hence, three of four hAEC and hAMSC strains at P2 clearly showed positive differentiation. Adipogenic differentiation of hAMSC was less reproducible, with two positive cases of four. Strikingly, none of the six hAEC strains tested during this study showed evidence of adipogenic differentiation, which seems to be in contradiction to recent reports [8, 22, 23]. In 't Anker et al. published adipogenic differentiation of a stem cell population derived from mechanical disaggregation of the whole amniotic membrane and selection by completely different culture conditions compared to ours [8]. Portmann-Lanz et al. reported a transient growth retardation during which morphology changed from typically epithelial, cobblestone-like to a fibroblast-like morphology [23]. In our study, hAEC isolations with noticeable change of morphology towards the fibroblast-like phenotype were excluded to allow separate analysis of mesenchymal and epithelial cells. Discrepancies with published reports may be due to the use of different cell populations with different differentiation potential or the application of different culture conditions [8, 22, 23].

We further focused on a detailed characterization of the surface antigen expression profile of freshly isolated amniotic cells and its alteration during *in vitro* cultivation using hAEC. We demonstrate that hAEC undergo profound changes during the first days in culture, concomitant with, but not dependent on entry into the cell cycle. Intriguingly, several markers associated with MSC, such as CD90 and CD105, are expressed at low levels or not at all on primary isolates and are upregulated only after cultivation. This is of major importance considering possible immunoisolation methods of noncultured cells for stem cell enrichment, which has been established for $CD105^+$ bone marrow derived stem cells [8].

The observed alteration of the phenotype may be explained by several mechanisms. First, cells with the "altered" phenotype might be present in the primary isolate as a minor population and overgrow the main population due to a growth advantage *in vitro*; this explanation is highly implausible, as the change in surface antigen expression occurs very rapidly and hAEC have low growth rates *in vitro*. Second, the immunophenotype of the entire population might shift during cultivation or, third, only a subpopulation of the isolated cells might adapt to the culture conditions by changing its phenotype and become enriched by passaging. Directly after isolation, the population is heterogeneous with e.g., 20–50% being CD13-positive and the remaining 50–80% CD13-negative cells. We estimate that about two thirds of isolated hAEC become adherent during the first 3 days in culture, after which we usually remove the cells of the supernatant. Interestingly, preliminary results suggest that these nonadherent cells differ from their adherent counterparts, e.g., in reduced CD44 and increased CD54 expression (G. Stadler, unpublished observation). However, adherence to plastic does not trigger the full spectrum of alterations noticed after three passages, as expression of group 3 antigens is still rather low at day 3 (d3), when cells have just adhered to the culture dish (in Fig. 1d, CD105 and CD49e are shown as representative examples for group 3 antigen expression). Thus, we hypothesize that, as a first selection step, only part of the primarily isolated heterogeneous cell population adheres to plastic, concomitant with a partial upregulation of group 3 antigens that are further upregulated during continued *in vitro* cultivation to result in a homogeneous population with high expression of mesenchymal stem cell markers.

Finally, we have addressed the crucial question regarding the consequences of the phenotypic shift during cultivation in terms of differentiation capacity, which has to be answered before applying cultivated stem cells for tissue engineering. We found that osteogenic differentiation was reduced after cultivation for two passages, at a time point at which group 3 antigens were homogeneously and highly expressed and embryonic TRA-antigens (group 4) were reduced to undetectable levels. Functional impairment through cultivation has also been shown for primary murine BMSC, which lost their homing ability *in vitro* [82].

In conclusion, our results suggest that freshly isolated hAEC have a superior differentiation potential compared to hAEC cultivated under standard conditions and therefore we are currently aiming to develop culture conditions allowing maintenance of the original phenotype and differentiation capacity.

6.3 Platelet Lysate for FCS-Free Expansion and Cryoconservation of Amnion-Derived Cells

PL is an interesting alternative to FCS during expansion and cryopreservation of cells intended for application in humans. Expansion of hAMSC in a PL containing medium is superior to a medium containing FCS. However, cryopreservation in PL decreases cell viability after thawing. For future cell banking attempts, a combination of expansion in PL medium and cryopreservation in serum free cryomedium may allow for animal free strategy for expansion, cultivation and banking of hAMSC. However, some pitfalls including low attachment capacity of PL-expanded hAMSC have to be overcome.

6.4 hTERT Induced Extension of In Vitro Life Span of Amnion-Derived Stem Cells

The applied strategy was successful in creating immortalized cell lines with largely retained characteristics of the parental cells with regard to morphology, surface marker profile, and immunosuppressive capacity and showed similar or improved differentiation potential. However, one of two hAMSCtelo lines resulted in a significantly higher immunogenicity compared to the nontransduced controls, although the surface marker profile currently regarded as most important for human MSC characterization did not differ from the parental cells. This suggests that yet unknown markers will have to be identified in order to predict immunogenicity of the cells. In summary, the novel cell lines give proof of principle that hTERT is a promising tool to generate sufficient material for stem cell banking and tissue engineering, but concomitantly emphasize the need for careful and standardized characterization.

Stem cell characteristics of the newly established cell lines, especially their differentiation and immunogenicity, were variable. Concerning typical surface marker profiles, hAMSC lost the mesenchymal marker CD90 in a subpopulation of telomerized cells during prolonged *in vitro* propagation. It has recently been published that, upon cultivation in EGM-2 (also used in our study for hAMSC), a subpopulation of CD90 negative human BMSC evolved after prolonged culture, probably due to angiogenic growth factors in the medium [83]. The decrease in CD90 expression and concomitant increase of the embryonic stem cell marker SSEA-4 found in our immortalized hAMSC lines suggests an alteration of the phenotype during long-term culture in EGM-2.

Both parental hAMSC isolates possessed low adipogenic differentiation potential which has been described before [68, 69]. However, hTERT transduction led to increased lipid accumulation of hAMSC76 under adipogenic conditions. This is in contrast to the finding that mesenchymal stromal cells from chorion cotransduced

with hTERT and Bmi-1 showed minimal adipogenic differentiation that even decreased with time in culture [84].

We observed immunosuppression of activated PBMC by our immortalized cell lines, the exception being hAMSC76telo. Since the cell surface marker profiles of hAMSCtelo lines analyzed here were identical, we propose that a marker as predictor for immunogenicity remains to be identified and included in routine surface marker profiling.

In conclusion, the immortalized stem cell lines established in this study can be seen as a first step to a proof of principle for their applicability in cell based therapy approaches. Since obvious donor and cell line specific differences exist, stem cell material for cell banks will have to be routinely tested. Specifically, their differentiation potential and immunosuppressive effects are of major importance. However, additional caveats that limit the use are controversially discussed in the literature. In particular, the tumorigenic potential of stem cells in general and hTERT-transduced cells specifically is a matter of debate. Most reports find that stem cells retain their differentiation potential, contact inhibition properties, stable karyotype, and do not show tumorigenic potential even after extensive *in vitro* expansion. In other studies, spontaneous transformation of human ASC after 4–5 months in culture and a high rate of tumorigenicity evolving as a consequence of hTERT introduction in human MSC after approximately 3 years in culture were reported [2, 85]. Hence, it can be expected that given a high quality of starting material concerning stem cell characteristics and genetic stability and by use of a reasonable culture time *in vitro* suitable stem cell material can be made available for cell banking by careful monitoring and characterization.

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Mesenchymal Stromal Cells Derived from Human Umbilical Cord Tissues: Primitive Cells with Potential for Clinical and Tissue Engineering Applications

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Abstract Mesenchymal stem or stromal cells (MSCs) have a high potential for cell-based therapies as well as for tissue engineering applications. Since Friedenstein first isolated stem or precursor cells from the human bone marrow (BM) stroma that were capable of osteogenesis, BM is currently the most common source for MSCs. However, BM presents several disadvantages, namely low frequency of MSCs, high donor-dependent variations in quality, and painful invasive intervention. Thus, tremendous research efforts have been observed during recent years to find alternative sources for MSCs.

In this context, the human umbilical cord (UC) has gained more and more attention. Since the UC is discarded after birth, the cells are easily accessible without ethical concerns. This postnatal organ was found to be rich in primitive stromal cells showing typical characteristics of bone-marrow MSCs (BMSCs), e.g., they grow as plastic-adherent cells with a fibroblastic morphology, express a set of typical surface markers, and can be directly differentiated at least along mesodermal lineages. Compared to BM, the UC tissue bears a higher frequency of stromal cells with a higher in vitro expansion potential. Furthermore, immune-privileged and immune-modulatory properties are reported for UC-derived cells, which open highly interesting perspectives for clinical applications.

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Keywords Counterflow centrifugal elutriation, Mesenchymal stem cell, Mesenchymal stromal cell, MSC, Umbilical cord

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Abbreviations

ALP	Alkaline phosphatase
b-FGF	Basic fibroblast growth factor
BM	Bone marrow
BrdU	5-Bromo-2-deoxyuridine
CCE	Counterflow centrifugal elutriation
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CFU-F	Colony forming unit-fibroblast
DAPI	4′,6-Diamidino-2-phenylindole
ESC	Embryonic stem cell
GMP	Good manufacturing practice
GvHD	Graft-versus-host disease
HA	Hyaluronic acid
HLA	Human leukozyte antigen
HUCPVC	Human umbilical cord perivascular cells
ISCT	International Society for Cellular Therapy
MLC	Mixed lymphocyte culture
MSC	Mesenchymal stromal cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
UC	Umbilical cord
UCB	Umbilical cord blood
UCMS	Umbilical cord matrix cells
VEGF	Vascular endothelial growth factor
WJ	Wharton's jelly
WJC	Wharton's jelly cell

1 Introduction

The engineering of stem cells to restore defect functions in human tissues is an exciting challenge in the field of regenerative medicine. Embryonic stem cells (ESC) have a great potential for this purpose due to their pluripotent differentiation capability, but their use is limited by serious ethical considerations. Recent findings evidencing the reprogramming of somatic cells to pluripotent stem cells (termed IPS cells) [1] open ethically acceptable perspectives. However the concern remains that undifferentiated IPS cells as well as ESC may form teratomas after transplantation in the body.

Adult mesenchymal stem or stromal cells (MSCs) are considered a valuable alternative to these cells. Since their discovery in bone marrow (BM) by Friedenstein et al. [2], BMSCs have been extensively investigated and their use in animal studies as well as in clinical trials showed encouraging results (reviewed in [3]). Today BM-MSCs are still considered as the "gold standard" for the use of adult MSCs. Nevertheless BM as a source for MSCs presents several disadvantages. Besides the invasive and painful collecting procedure, in BM-aspirates MSCs are present at very low frequency (approximately 0.001–0.01% [4]) and their quality varies with the age of the donor. The low frequency implies that an extensive in vitro expansion of the cells will be required to deliver clinical doses to a patient, which enhances the risk of epigenetic damages as well as viral and bacterial contaminations. For these reasons, alternative sources of MSCs are needed.

In this context, the human umbilical cord (UC) gained more and more attention during the last decade (see Fig. 1). The UC is a non-controversial and accessible source of autologous cells, which can be easily processed after birth. It has been demonstrated that MSCs are found both in the blood (UCB) [5] and in the tissues of UC. However, UCB-derived MSCs may have a limited technological potential because their frequency seems even lower than in BM (range 0.001–0.000001% [6]) and their isolation is hardly reproducible [7, 8], whereby UCB contains larger amounts of other tissue stem cell populations including $CD133^+$ cells or hematopoietic stem cells ($CD34^+$). In contrast, the frequency of MSCs in UC-tissues is believed to be much higher. Thus, using robust isolation procedures, a large number of multipotent primitive stromal cells with high proliferation capacity can be isolated. All these features open interesting perspectives for the scalable production and engineering of UC-derived cells for clinical applications. Here, we give an overview of the scientific evidences collected during the recent years that human UC may be a valuable cell source for cell-based therapies.

2 The Human Umbilical Cord: A Source of MSCs

The human UC is the lifeline between the fetus and the placenta. It is formed during the fifth week of embryogenesis and grows to a final length of approximately 60–65 cm, weighs about 40 g, and has a mean diameter of 1.5 cm in normal

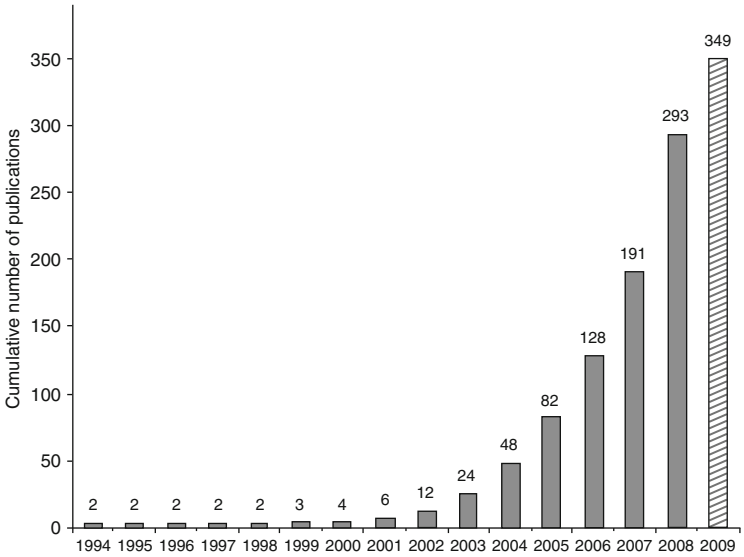

Fig. 1 Cumulative number of publications over the last 15 years dealing with UC-derived MSCs (entries by PubMed with the terms "mesenchymal stem cells" and "umbilical cord" till July 2009)

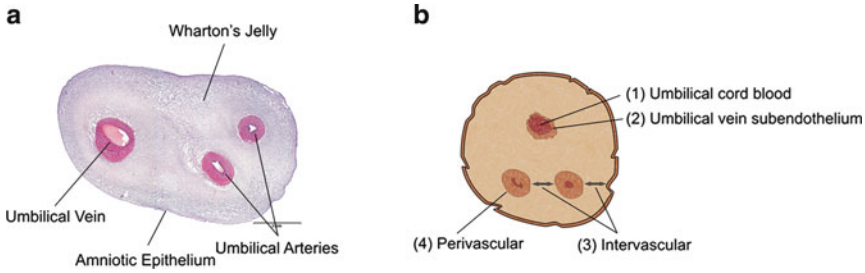

Fig. 2 Cross section of an umbilical cord. (**a**) UC consists of two arteries and one vein embedded in the Wharton's jelly and surrounded by an amniotic epithelium (modified from [95]). (**b**) Four separate compartments within the umbilical cord have been shown to comprise mesenchymal stromal cells

pregnancies [9, 10]. UC usually comprises two arteries and a vein, which are immersed within the so-called Wharton's jelly (WJ) and enclosed by a simple amniotic epithelium (see Fig. 2a). WJ is a mucoid connective tissue rich in proteoglycans and hyaluronic acid (HA), which insulates and protects umbilical vessels from torsion, compression, or bending and therefore ensures a constant blood flow between fetus and placenta.

In recent years, several studies described at least four separate regions (see Fig. 2b) of the UC containing MSCs. The term "MSC" has been related to several definitions. In this chapter we use "MSC" as an acronym for mesenchymal stromal cell (discussed in Sect. 4). MSCs could successfully be isolated from UCB [7, 11–15], the umbilical vein subendothelium [16–18], the intervascular region [19–29], the perivascular region [30, 31], or from whole UC tissue [32, 33]. UC-derived MSCs meet the basic definition of multipotent MSCs as postulated by the International Society for Cellular Therapy (ISCT) (see Sect. 4).

Thus, this chapter will focus on MSCs derived from UC tissue, but not from UCB (see Fig. 2b, region 2–4).

3 Isolation of MSCs from the Umbilical Cord

In recent years, several investigators published protocols for isolating MSCs from the UC tissue. Depending on from which part of the cord the cells should be isolated, protocols have been adopted and modified. A schematic overview of applied isolation protocols is given in Fig. 3. Basically, the isolation procedure starts with the removal of umbilical vessels. The cord is then cut down to smaller segments or chopped into small pieces which are subsequently enzymatically digested [22, 23, 29]. Alternative isolation methods without removal of vessels [34, 35] and without enzymatic digestions [26, 34] or explant cultures [33, 36] have also been described. To isolate cells from the perivascular tissue or the subendothelium of the umbilical vein, further methods have been established [16–18, 30, 31].

We have used a protocol without enzymatic digestion and without removal of umbilical vessels to isolate MSC-like cells from whole UC tissue in an explant culture approach. Therefore, human UCs were obtained from patients with written consent delivering full-term (38–40 weeks) infants by cesarean section. The use of this material has been approved by the Institutional Review Board, project #3037 in an extended permission on June 17, 2006.

First, blood from arteries and the vein was removed by flushing phosphate buffered saline (PBS) through the vessels using a sterile syringe and blunt needles. Thereafter, UC was stored in an appropriate transfer medium (PBS) enriched with 5 g L^{-1} glucose, 50 μg mL^{-1} gentamicin, 2.5 μg mL^{-1} amphotericin B, 100 $U.mL^{-1}$ penicillin, and 100 μg mL^{-1} streptomycin), to minimize the risk of contaminations. The UC was first cut into 10–15 cm long segments which were subsequently cut into approximately 0.5 cm^3 large pieces. During the isolation procedure, transfer medium was used to keep the cord and the minced pieces moist. Finally, the small pieces were transferred to cell culture flaks (Fig. 4a) and incubated in αMEM supplemented with 15% of allogous human serum and 50 μg m^{-1} gentamicin at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed every second day. An outgrowth of adherent cells from single tissue pieces could be observed after approximately 10 days (Fig. 4b). After 2 weeks the UC tissue was removed and the adherent cells (Fig. 4c) were harvested by enzymatic treatment. The obtained cell suspension was

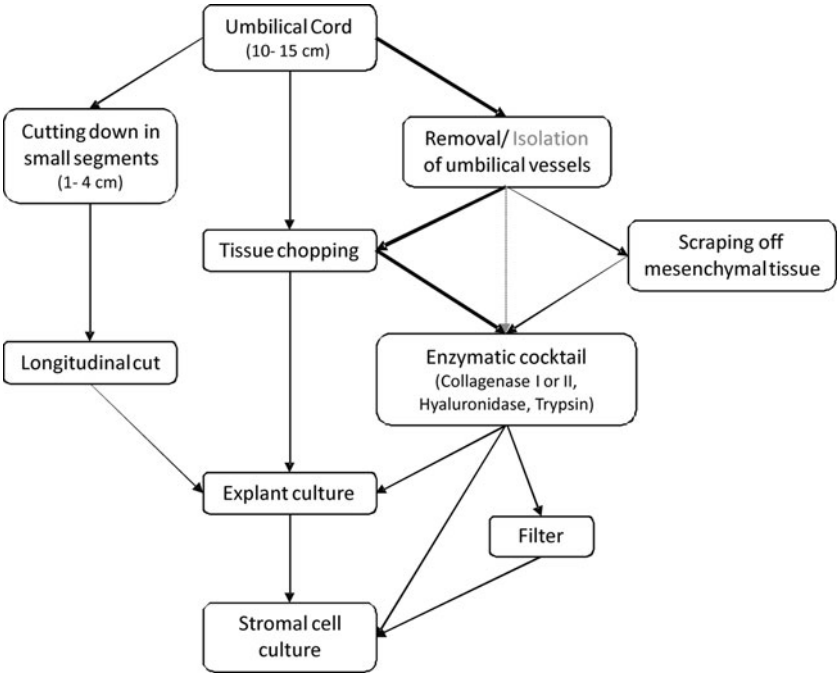

Fig. 3 Schematic overview of applied isolation protocols. Various approaches have been used to isolate mesenchymal stromal cells from the umbilical cord tissue. Basically, the isolation procedure includes steps of removing umbilical vessels, tissue chopping and enzymatic digestion (indicated by *bold arrows*), but several alterations of protocols have been described

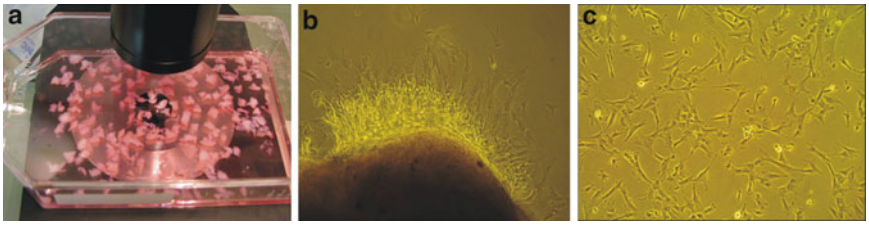

Fig. 4 Isolation of mesenchymal stem cell-like cells from umbilical cord. (**a**) Explant culture of minced UC tissue. (**b**) After approximately 10 days of culture cells start to grow out of the small UC segments. (**c**) Adherent growing monolayer of fibroblast-shaped cells after 2 weeks of culture

centrifuged at 200 g for 5 min and the cells were resuspended in αMEM supplemented with 10% human serum and 50 μg mL^{-1} gentamicin and subcultured at a density of 4000 cells cm^{-2}. These culture conditions have demonstrated to support an optimal growth of the cells.

The isolated cells exhibited a high proliferation potential. Cell population doubling times ranged from 27.5 ± 1.6 h (passage 2) to 78.9 ± 6.3 h (passage 17).

At our culture conditions, the cells could be expanded without loss of proliferative activity and viability at least for 20 population doublings. After approximately 50 population doublings the cells entered a phase of replicative senescence. High proliferation potential and expansion capacity are common features for UC-derived stromal cells, which were described by several other groups [20, 26, 29, 30, 34, 35, 37, 38]. Furthermore, UC-derived cells could be efficiently cryopreserved and revitalized. We used a cryo-medium containing 80% human serum, 10% culture medium, and 10 % DMSO. The cells were gradually frozen at a rate of 1°C min^{-1} and finally stored at −196°C. At these conditions cell survival rate after rapid thawing at 37°C reached 75 ± 12.8%.

To date, it still remains to be further investigated whether cells isolated from different compartments or derived by different isolation procedures share the same stem cell characteristics, e.g., proliferation and differentiation potential and immunologic properties (see below).

4 Characterization of UC-Derived MSCs

The acronym "MSC" has been widely used in the literature for "mesenchymal stromal cell" as well as for "mesenchymal stem cell" to denominate plastic-adherent fibroblast-like cultures isolated from different adult or extra-embryonic tissues. Because there is currently no consensus set of markers allowing the identification of MSCs and considering the fact the definition criteria for stem cell is not unanimously accepted [39], it appeared unwise to apply the term "stem cell" for mesenchymal cell population. In this context, ISCT proposed to term plastic-adherent fibroblast cultures "multipotent mesenchymal stromal cells" (MSC) [40] and published in 2006 the minimal criteria defining these cells [41].

UC-derived stromal cells meet the basic criteria defined by the ISCT, namely the adherence to plastic, the expression of a set of specific surface antigens (see below), and a multipotent differentiation potential (discussed in paragraph 5 Differentiation Potential).

Histologically, cells freshly isolated from the UC are mainly fibroblastic in appearance (see Fig. 5a). However, some groups reported more than one phenotype in UC-derived MSC cultures [23, 29, 31, 36] and noticed changes in the distribution of the phenotype after several passages [23, 36]. Our group observed for instance a broad cell size distribution and marked morphological differences in isolated UC-MSCs cultures (see Fig. 5b). After fractionation of different populations via counterflow centrifugal elutriation (CCE) according to the size of the cells, we obtained two sub-populations with significant differences in cell size, growth properties, and biochemical markers expression. Whereas small-sized subpopulation exhibited the highest proliferative capacity and the most pronounced expression of MSC markers, large-sized cells were identified as senescent via β-galactosidase staining (see Fig. 6) [36]. These findings may be of importance in order to deliver high quality cells for clinical applications.

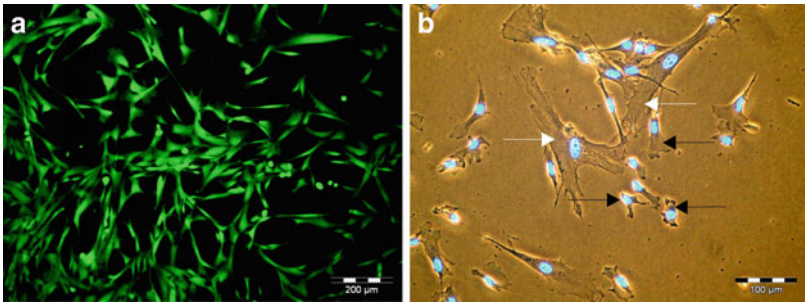

Fig. 5 Morphology of UC-derived stroma cells. (**a**) UC MSC show predominantly fibroblastic morphology. The cytoplasm of cells was visualized via Calcein-AM stain (100× magnification). (**b**) Cells with marked morphological differences can be rather observed in the cultures collected from UC. The image presents large cells (a, *white arrows*) surrounded by small cells (b, *black arrows*), which show increased nucleus-to-cytoplasm ratio. For the visualization of cell nuclei DAPI staining was performed (200× magnification)

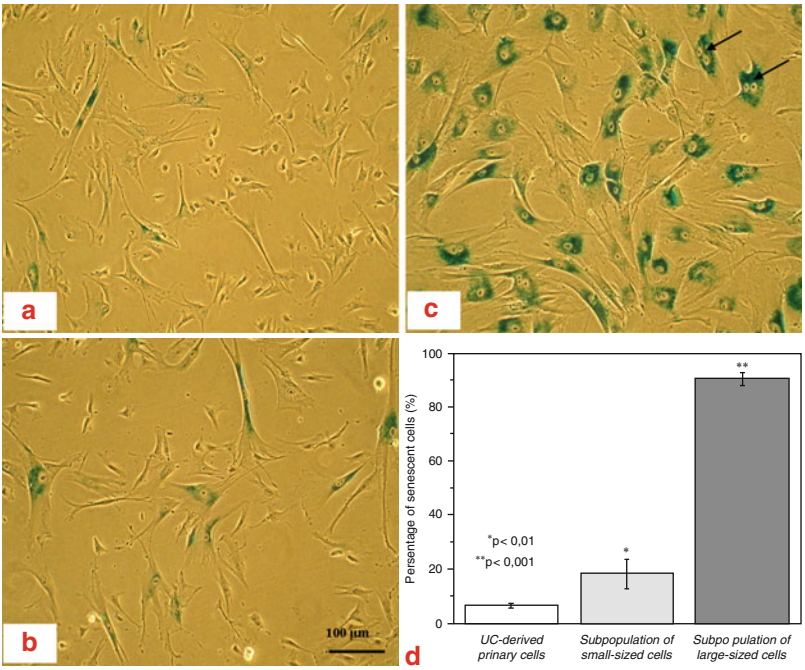

Fig. 6 Senescence staining in sub-population of UC-derived primary cell cultures [36]. (**a**) UC-derived primary cell population. (**b**) CCE-derived subpopulation of small-sized cells. (**c**) CCE-derived subpopulation of large-sized cells. (**d**) Comparison of the senescence level in the CCE-derived fractions. Cells were cultured for 6 days after elutriation. Following subculture, the cells were seeded at a density of 6000 cells cm^{-2} and cultured for further 48 h in complete medium. A relative high portion of multi-nucleated cells (*arrows*) were detectable in the subpopulation of the large-sized cells. Student's *t*-tests were performed for the recognition of the significant differences (marked with *asterisks*) in comparison to UC-derived primary cell population

An additional feature of MSCs is their clonogenicity. A single cell is able to rise to a fibroblastic colony in a so-called colony forming unit fibroblast (CFU-F) assay. Historically, this characterization parameter is linked to the pioneer work of Friedenstein et al., who first isolated stromal cells from BM according to their capability to form fibroblastic colonies and demonstrated their osteogenic potential in vitro [2]. The CFU-F assay gives the frequency of fibroblast-like cells within a population liable to extensive proliferation and to rise to a colony (see Fig. 7).

This approach is commonly used to enumerate MSCs in a particular tissue [4]. For instance, Lu et al. recently evaluated the frequency at 1 CFU-F per 1609 mononuclear cells (MNCs) in whole UC tissues [35]. More specifically, 1 CFU-F per 333 MNCs was reported in cells isolated from perivascular tissues of the UC vein [31]. In comparison, the isolation frequency of CFU-F from BM is estimated in a range of 1–10 CFU-F per 10^5 MNCs [4] and only 1 CFU-F per 10^8 MNCs [7] to 1–3 CFU-F per 10^6 MNCs are reported in UCB [42, 43]. According to these data, the human UC is considered to harbor a higher number of MSCs than found in BM or UCB. The results of the CFU-F assay, however, depend on different parameter such as the isolation method, culture conditions, as well as the cell seeding density. This leads to a high degree of variability in the results and makes the comparison of the published data difficult. Analysis of specific molecule expression at the single cell level via flow cytometry is strongly advisable to identify MSCs within a mixed cell population.

In contrast to other progenitor cell populations, such as, for instance, hematopoeitic stem cells, there is currently no specific marker available defining human MSCs. The expression of a set of markers combined with the demonstration of in vitro multi-lineage differentiation potential is necessary to identify MSCs in UC-derived cell populations. Table 1 summarizes extracellular and intracellular molecules expressed by UC-MSCs reported in the literature up to July 2009.

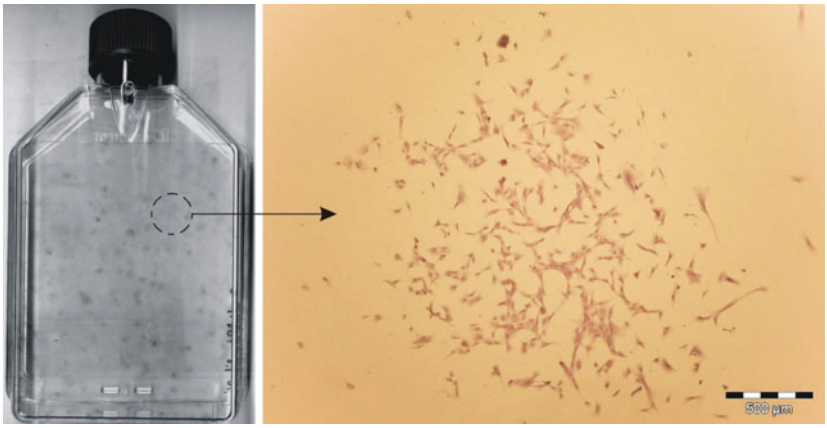

Fig. 7 CFU-F assay of UC-derived stromal cells

Table 1 Reported intra- and extra-cellular markers of UC-derived MSCs till July 2009

Marker	Expression	References
CD10	+	[24, 29]
CD13	+	[24, 29, 35, 54, 96–101]
CD14	–	[23, 24, 29, 35, 97, 102, 103]
CD29 (integrin β1)	+	[28, 29, 35, 54, 55, 66, 100, 102–106]
CD31 (PECAM)	–	[29, 30, 35, 54, 66, 100, 105]
CD33	–	[29, 60]
CD34	–	[20, 23, 24, 28–31, 35, 36, 54, 55, 60, 66, 97, 98, 100–103, 105, 106]
CD38	–	[20, 100, 106]
CD44	+	[23, 24, 28, 29, 31, 35, 36, 54, 60, 66, 98, 100, 101, 103, 105, 106]
CD45	–	[20, 23, 24, 28–31, 35, 36, 54, 60, 66, 98, 100, 101, 103, 105]
CD49b (integrin α2)	+	[29, 98]
CD49c (integrin α4)	+	[29]
CD49d (integrin α3)	+	[29]
CD49e	+	[29, 30]
CD51 (integrin α5)	+	[21, 28, 29]
CD54 (ICAM-1)	–/+[a]	[20, 98, 105]
CD56	–	[29]
CD71	–/+	[103, 106]
CD73 (SH3)	+	[21, 23, 24, 28, 31, 35, 36, 45, 55, 66, 101–105]
CD90 (Thy-1)	+	[20, 24, 29–31, 35, 36, 45, 54, 55, 60, 66, 98, 101, 103, 104]
CD105 (endoglin, SH2)	+	[20, 21, 23, 24, 28, 29, 31, 35, 36, 45, 60, 66, 97, 98, 100, 102, 105]
CD106 (VCAM-1)	–/+[a]	[31, 35, 54, 56, 98, 107]
CD117 (c-kit)	–/+[a]	[24, 26, 30, 31, 45, 54, 60, 98, 103, 104]
CD123 (IL-3 receptor)	–	[31]
CD133	–	[29]
CD146	+	[30, 56, 108]
CD166 (ALCAM)	+	[35, 45, 101, 102, 104, 105, 109]
CD235a (glycophorin A)	–	[31]
CD271	ND	
Bmi-1	+	[89, 106]
Esrrb	–	[89]
GD2	+	[47]
HLA-1	+	[20, 29, 48, 106]
HLA-DR (MHC class II)	–	[24, 29, 31, 33, 35, 54, 55, 66, 98, 100, 101, 104, 106, 107]
HLA-DP (MHC class II)	–	[24, 31, 100, 103]
HLA-DQ (MHC class II)	–	[24, 31, 103]
HLA-A, B, C (MHC class I)	+	[24, 31, 35, 54, 98, 101–104]
HLA-G (MHC class I)	–/+[a]	[31, 34, 82]
Hoxb-4	–	[89]
MSCA-1	n.d.	
Nanog	+	[29, 45, 47, 48, 89, 110]
Nucleostemin	+	[89, 106, 110]
Oct-3/4	–/+[a]	[34, 45, 47, 48, 89, 110, 111]
Rex-1	+	[45]
Sox-2	+	[29, 45, 47, 110]

(*continued*)

Table 1 (continued)

Marker	Expression	References
SSEA-3	–/+[a]	[45, 89]
SSEA-4	–/+[a]	[31, 45, 47, 66, 89]
STRO-1	–/+[a]	[30, 31, 48]
Tbx-3	–	[89]
TCL-1	–	[89]
Tra-1-60	–/+[a]	[45, 89]
Tra-1-81	–/+[a]	[45, 89]
ZFX	+	[89]
Zic-3	–	[89]

[a]Discrepancy among the published results

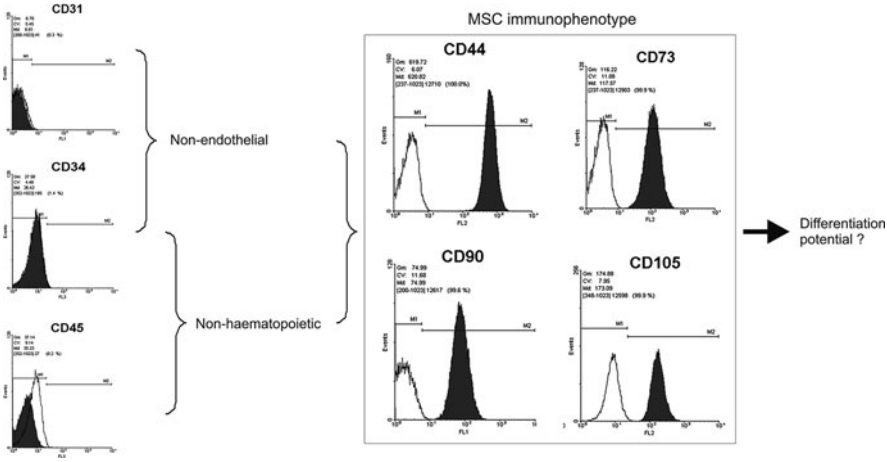

Fig. 8 Flow cytometric analysis of UC-derived stroma cells

The surface antigen SH2 (CD105), SH3 (CD73), and Thy-1 (CD90) are widely used for the identification of UC-derived stromal cells (see Table 1), as these markers are proposed by the ISCT as positive markers for human MSCs [41]. However, these epitopes are also expressed on hematopoietic and endothelial cells, which are two potential contaminants in UC-derived cell populations. Consequently, it is necessary to carefully exclude cells from hematopoietic or endothelial origin using surface marker such as CD45, CD34, or CD31. HA receptor CD44 is also a commonly accepted marker, as the extracellular matrix of the UC is one of the highest HA-containing tissue in humans [44]. Figure 8 exemplarily illustrates the immunophenotype of a stromal cell population isolated from whole UC tissue by our group. Additionally, like MSCs isolated from other tissues, UC-derived stroma cells do not express the human leukocyte antigen HLA-DR but express HLA-I. However, Sarugaser et al. reported that the expression of the latter marker may be manipulated in vitro, which may be very promising in term of allogenic transplantations [31].

UC-derived stroma cells were found positive for pluripotency markers usually expressed by ESCs such as Oct-3/4, Nanog, Sox-2, or SSEA-4 (see Table 1), which underlines their primitive nature. The primitive character of the UC-derived cells is also illustrated by their high proliferation and expansion capacity. UC-derived stroma cells have shorter doubling times compared to adult BM-MSCs [30, 35, 37, 38], exhibit telomerase activity [23, 26, 45], and could be expanded in vitro to a number of population doublings ranging from 20 to 80 without evidences of senescence or abnormal karyotype [20, 26, 29, 34]. It was first unclear whether UC-derived stroma cells were homogenous regarding their primitiveness or if UC-derived stroma populations rather harbor a subset of primitive MSCs [46]. For instance, population doubling times estimated between 60 and 85 h for freshly isolated UC-cells rapidly decrease within 2–3 passages to approximately 25 h [23, 31], which may indicate the presence of a fast growing sub-population of more primitive cells overgrowing the initial population. This hypothesis was further strengthened by recent works demonstrating via flow cytometry a subset of cells expressing pluripotency markers [47, 48]. Zhang et al., for instance, reported that approximately 20% of stroma cells isolated from perivascular tissues of the umbilical arteria express Oct-3/4 and Nanog [48].

With growing evidence that MSC-like cell population isolated from UC tissues are rather heterogeneous, at least in regard to primitive marker expression, the identification of a universal marker defining primitive human MSCs remains challenging. Several cell surface molecules were recently proposed for the identification and isolation of MSCs in BM aspirates such as CD271 [49, 50], MSCA-1 [50], SSEA-4 [51], and the neural ganglioside GD2 [52, 53]. To our knowledge, CD271 and MSCA-1 expressions have not been reported yet in UC-derived stroma cell populations. Xu et al. recently isolated a subset of $GD2^{+}$ cells exhibiting a high clonogenicity as well as proliferation capacity but also a significantly stronger multi-differentiation potential than $GD2^{-}$ cells. According to these results, GD2 may be a useful marker to isolate multipotent MSCs from UC-tissues, but further studies are needed to verify these findings.

The most convincing biological property for the identification of MSCs remains the capability to differentiate into mesodermal lineages. In the next section the in vitro differentiation potential of UC-derived stromal cells will be discussed.

5 In Vitro Differentiation Potential

The differentiation repertoire of stroma cells derived from UC tissue reported in the literature till July 2009 is summarized in Table 2.

The potential of UC stroma cells to differentiate into adipocytes, chondrocytes, and osteocytes has been widely investigated and well established by several groups. According to the minimal definition criteria proposed by the ISCT, UC-derived stroma cells are considered multipotent MSCs [41]. Successful adipogenic, chondrogenic, and osteogenic differentiation of UC-derived MSCs are presented in Fig. 9.

Table 2 Differentiation potential of stroma cells derived from human umbilical cord tissue reported in the literature till July 2009

	Cell type	References
Mesodermal lineage	Adipocyte	[16, 17, 20, 23, 28, 30, 34, 35, 37, 45, 48, 54–56, 60, 63, 67, 102, 103, 105–108, 112, 113]
	Chondrocyte	[17, 19, 23, 28, 30, 37, 45, 48, 55, 56, 60, 63, 67, 102, 103, 108, 112]
	Osteocyte[a]	[16–18, 20, 23, 28, 30, 31, 34–37, 45, 48, 54–56, 60, 63, 66, 67, 102, 103, 105–108, 112–115]
	Cardiomyocyte[a]	[28, 56, 57, 105]
	Skeletal myocyte	[20]
	Endothelial cells	[37, 54, 55]
Ectodermal lineage	Neuronal cells	[21–23, 25, 26, 35, 60–62]
Endodermal lineage	Islet-like cells	[38, 59]
	Immature hepatocyte	[58]

[a]Discrepancy among the published results

Adipogenic potential is usually demonstrated by the apparition of cells exhibiting intracellular lipid droplets (Fig. 9a). The capacity to form chondroblasts is evidenced by the formation of shiny cell-spheres with type II collagen expression in the extracellular matrix in droplet cultures (Fig. 9b). Enhanced ALP expression and mineralization assayed by von Kossa or alizarin red staining demonstrate osteogenic potency (Fig. 9d, e). It should also be mentioned that sub-populations of cells spontaneously exhibiting a functional osteogenic potential with mineralized bone nodules can be observed in UC-MSCs cultures [31]. Such bone nodules are presented exemplary in Fig. 10.

In addition, it has been shown that UC-MSCs can successfully differentiate to endothelial cells after addition of VEGF and b-FGF [54, 55] and can form vessel-like structures in matrigel cultures [37, 55]. Furthermore, some UC-derived cell populations also seem to be able to differentiate to muscle cells. For instance, WJ cells (WJCs) could be induced to skeletal myocytes when placed in myogenic medium [20]. Differentiation to cardiomyocytes was also reported but remains controversial. Whang et al. demonstrated for instance that WJCs could be induced to cells exhibiting cardiomyocyte morphology and expressing specific markers (*N*-cadherin and cardiac troponin) using 5-azacytidine or cardiomyocyte-conditioned medium [28]. Kadivar et al. observed cardiomyocyte like cells expressing cardiac specific genes after 5-azacytidine induction of UC-MSCs isolated from the endothelium/subendothelium layer of the UC vein. In contrast to these results, Martin-Rendon et al. could not detect cardiac markers expression after in vitro induction of MSCs isolated from the WJs and perivascular tissues [56]. Furthermore, differentiated in vitro cultures of functional cardiomyocytes presenting beating clusters are poorly or not demonstrated. To our knowledge, only one group reported differentiated cells exhibiting slight spontaneous beating after 21 days of induction; however no quantitative data are presented in this study [57].

Recent findings suggest that UC-MSCs can differentiate into endodermal lineages. Campard et al. reported that UC-matrix cells constitutively expressed

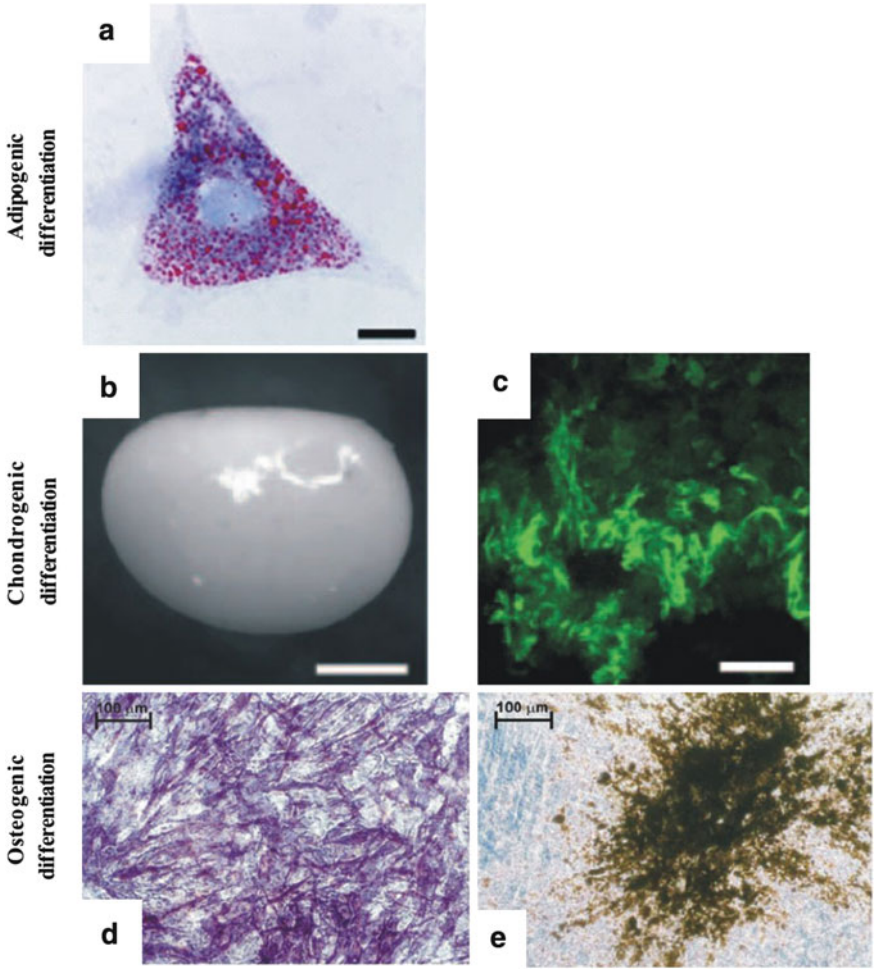

Fig. 9 Adipogenic, chondrogenic and osteogenic potential of UC-derived MSCs. (**a**) Formation of lipid droplets stained with oil red O in Wharton's jelly cells after adipogenic induction, scale bar = 20 µm (modified from [23]), (**b**) cell sphere obtained in droplet culture of chondrogenically induced UC-MSCs (scale bar = 500 µm) with abundant type II collagen expression (in **c**, scale bar = 50 µm) (modified from [23]), (**d**) ALP expression after osteogenic differentiation of umbilical vein derived MSCs (modified from [17]). (**e**) Mineralization of osteogenically induced culture of umbilical vein derived MSCs evidenced by von Kossa staining (modified from [17])

markers of hepatic lineage, such as albumin, alpha-fetoprotein, cytokeratin-19, connexin-32, and dipeptidyl peptidase IV. After in vitro hepatic induction, cells exhibiting a hepatocyte-like morphology with hepatic features such as specific markers up-regulation and urea production were observed. However, the authors pointed out that their cells lack important characteristics of functional liver cells and thus conclude that UC-matrix cells can be differentiated at least to immature hepatocytes [58]. Chao et al. were also able to induce WJCs using a four stage

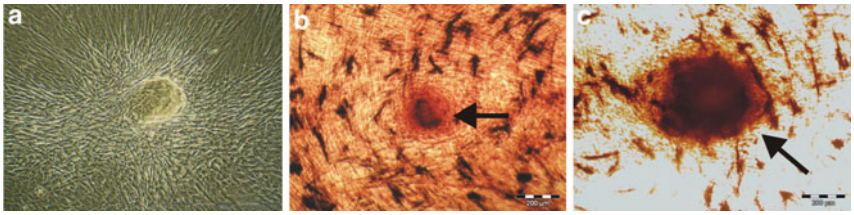

Fig. 10 Mineralized bone nodule in UC-MSCs culture. (**a**) Phase contrast microscopy picture of a bone nodule, (**b**) alkaline phosphatase (*violet dark cells*) and alizarin red staining of a nodule (*arrow*), (**c**) alkaline phosphatase (*violet dark cells*) and von Kossa staining of a nodule (*arrow*)

differentiation protocol to form islet-like clusters expressing pancreatic related genes and secreting insulin in response to glucose concentrations [59]. Recent results from Wu et al., who successfully differentiated WJCs to pancreatic cells and observed higher differentiation potential compared to BM-MSCs [38], further reinforce these findings.

Finally, several groups observed the differentiation of WJCs to cells exhibiting morphological and biochemical characteristics of neural cells, suggesting that UC-MSCs are able to differentiate to a certain state of maturation along the neuronal lineage [21–23, 25, 26, 35, 60–62]. Mitchell et al. were the first to observe neuronal differentiation of WJCs after stimulation with b-FGF and other neuronal differentiation reagents [26]. The differentiation was attested according to morphological changes and expression of neuron-specific enolase, βIII-tubulin, neurofilament M and tyrosin hydroxylase [26]. The differentiation potential was then confirmed by several other groups [21, 23, 25, 60]. Figure 11 shows exemplary neuronal cells obtained by Karahuseyinoglu et al. after neuronal induction of a sub-population of WJCs [23]. Interestingly, it also seems possible to generate some sub-types of neurones as demonstrated by Fu et al., who were able to obtain dopaminergic neurones from WJCs [21].

Summarizing the published data, we find strong evidence to suggest that the human UC is a source of multipotent stroma cells which are capable of differentiating into mesodermal and non-mesodermal lineages. It remains unclear whether the differentiation potential of the UC-derived MSCs depends on their location in the UC-tissues. For instance, Suzdal'tseva et al. reported that only a few cells isolated from the cord vein subendothelial tissue were able to differentiate to osteoblasts [63]. In contrast, cells isolated from perivascular tissues of the umbilical vein showed a high osteogenic potential with spontaneous formation of bone nodules [31], which was even evaluated higher than the potential of bone-marrow MSCs in a comparative study [30]. Recently, two sub-populations were evidenced in cultures of WJ-derived MSCs with regard to the expression of vimentin and pan-cytokeratin filaments [23]. Interestingly, cells expressing cytokeratin, predominantly located in the perivascular tissue of the cord, did not differentiate into neurones in vitro. These findings are consistent with the results of Sarugaser et al., who showed that perivascular UC-cells could not be induced to the neuronal lineage [31].

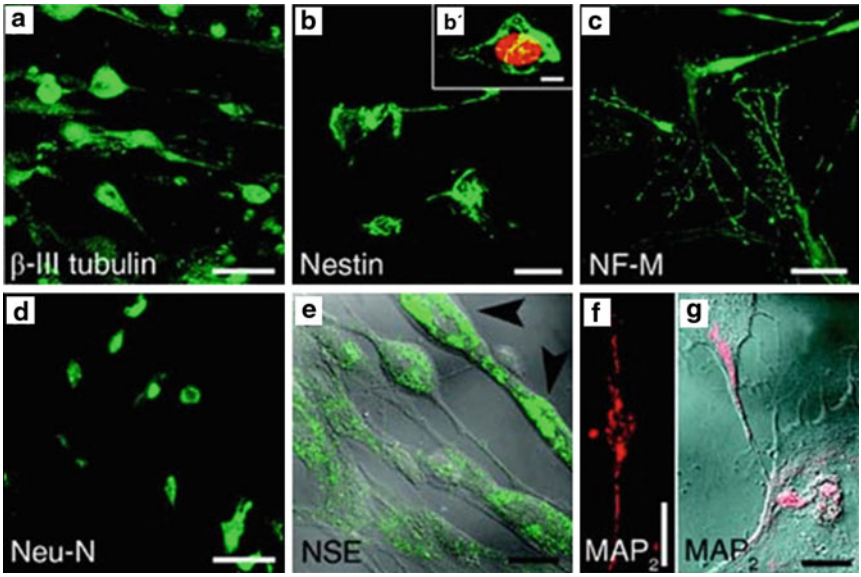

Fig. 11 Neuronal differentiation of WJCs, modified from [23]. (**a**) β-III Tubulin expression, (**b**) Nestin expression located in the perinuclear cytoplasm in particular (**b′**), (**c**) neurofilament-160 (NF-M), (**d**) neuron-specific nuclear protein expression (Neu-N) restricted to the nucleus, (**e**) neuron-specific enolase (NSE), (**f**) microtubule-associated protein-2 (MAP2) detected as discontinuities along the cells. (**g**) MAP2 distribution in cell–cell contact. *Scale bars* = 10 μm (**b′**), 20 μm (**b**, **c**), 50 μm (**e**), 100 μm (**a**, **c**, **d**)

The hypothesis of a location-dependent differentiation potential of UC-derived stroma cells is also supported by the fact that a gradient of cell maturity was observed within the UC tissues [64]. According to the cytoskeletal complexity, the most immature cells are located in subamniotic and intervascular regions, whereas cells of perivascular regions may represent a more differentiated state [64, 65].

Many groups most likely investigate mixed populations of UC-MSCs, particularly if the cells are derived from whole UC or from the WJ. Thus, the results of studies comparing the differentiation potential of UC-derived MSCs with other sources (for example BM) should be carefully interpreted [17, 56, 66, 67]. More work is needed to attest whether cells isolated from a defined compartment of the UC is more suitable for a specific differentiation lineage. This information would be of tremendous importance for clinical applications of UC-derived MSCs.

6 Immune Properties of MSCs and In Vivo Applications

Besides their multi-lineage differentiation potential, BM-derived MSCs have been shown to exhibit immune-privileged and immune-modulatory properties, which predestine them as ideal candidates for cell-based therapies. They fail to

induce proliferation of allogeneic lymphocytes in vitro and do not induce an immune response when used in allogenic mismatched animal experimental models [68–70]. Furthermore, they have regulatory effects on several cells of the immune system (e.g., T, B, dendritic, and natural killer cells) [71–77], prolong skin graft survival [78], and have been used in clinical applications to reduce acute and chronic graft-versus-host disease (GvHD) [79, 80]. Currently, three groups have investigated the in vitro immune properties of UC-derived MSCs and observed similar immunologic phenotypes to that of BM-MSCs. Ennis et al. [81] used cells isolated from the perivascular tissue of the UC [human UC perivascular cells (HUCPVC)] in one- and two-way mixed lymphocyte cultures (MLC) with resting or activated peripheral blood lymphocytes (PBL) to examine whether HUCPVCs induce or modulate proliferation of immune cells. Proliferation of PBLs was determined by measurement of 5-bromo-2-deoxyuridine (BrdU) or tritiated thymidine [^{3}H] incorporation. They could show that HUCPVCs did not induce allogenic lymphocyte proliferation but reduced the proliferation of alloreactive PBLs in a dose-dependent way. Weiss et al. [82] describe similar observations using WJ-derived cells termed UC matrix stroma (UCMS) cells. In co-culture experiments they could show that UCMS cells not only suppressed the proliferation of Con-A-stimulated rat splenocytes [measured by live cell counting, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay and carboxyfluorescein diacetate succinimidyl ester (CFSE)-assay] and activated human peripheral blood mononuclear cells (PBMCs) or purified T cells (measured by tritiated thymidine [^{3}H] incorporation) but also did not induce any proliferation of resting immune cells. Furthermore, flow cytometric analysis revealed the absence of the immune response-related surface antigens CD40, CD80, and CD86. Yoo et al. [83] compared the immune-suppressive effect of BM-MSCs and WJ-derived MSCs on phytohemagglutinin-induced T cell proliferation and report that both BM-MSCs and WJ-MSCs effectively reduced the proliferation of immune cells.

In vivo applications of UC-MSCs revealed further interesting attributes similar to BM-MSCs. Regarding their potential for cell-based therapy applications, UC-derived mesenchymal cells seem to support tissue repair by stimulating and modulating tissue-specific cells rather than differentiating into specialized cells. Yang et al. [84] reported a positive modulation of microglia and reactive astrocytes activities by UC-MSCs when transplanted into rats after complete transection of the spinal cord. They detected an elevated production of various cytokines around the lesion promoting spinal cord repair. Similar to these findings, Weiss et al. [29] hypothesized a supportive function of UC-MSCs mediated by various secreted trophic factors when used in a rodent model of Parkinson's disease. Referring to their preliminary work on porcine UC-derived MSCs, which were successfully transplanted into rat brains without triggering an immune response or being rejected [85], they then transplanted human UC-MSCs into brains of Parkinson's disease model rats without any immune-suppression. The transplanted cells did not produce brain tumors or a frank host immune rejection response. Furthermore, they significantly mitigated induced motor deficits [29]. Liao et al. [86] used UC-derived MSCs in a rodent stroke model and observed that the cells, injected into the rat

brain, survived for at least 5 weeks and reduced injury volume and neurologic functional deficits of rats after stroke. They assume angiogenesis-promoting properties of the cells by producing angiogenic cytokines. Koh et al. [87] also applied a rodent stroke model. They used MSCs isolated from the umbilical vein subendothelium and induced differentiation of the cells into neuron-like cells, as indicated by morphology, expression of neuronal cell markers, and secretion of neurotrophic factors, before transplantation into rats. Since the UC-MSCs were both morphologically differentiated into neuronal cells and able to produce neurotrophic factors, but had not become functionally active neuronal cells, the authors hypothesize that the observed improvement in neurobehavioral function might be related to the neuroprotective effects of UC-MSCs rather than to the formation of a new network between host neurons and the implanted cells. Analogical findings were reported by Lund et al. [24]. They suggested a supportive behavior of MSCs in a rodent model of retinal disease when UC-MSCs were shown to contribute to photoreceptor rescue. The cells did not transform into neurons but more likely secreted neurotrophic factors, as indicated by higher expression levels of these factors in vitro.

Besides their supporting properties, UC-MSCs were also shown to be easily genetically manipulated. Friedman et al. [88] and Kermani et al. [89] both transfected UC-tissue derived MSCs with a GFP-reporter gene and created a stable cell line. Considering the immune-privileged and immune-modulatory properties, the cytokine production and supportive functions in vivo and the ability to be easily transfected, UC-derived mesenchymal cells are promising candidates for cell-based therapies and clinical applications. Currently, there are first clinical trials aiming to demonstrate if human UC-MSC have in vivo immune-suppressive effects and can be used for GVHD treatment ["*Allogeneic Mesenchymal Stem Cell for Graft-Versus-Host Disease Treatment (MSCGVHD)*"; ClinicalTrials.gov Identifier: NCT00749164; www.ClinicalTrials.gov].

7 Future Perspectives

In terms of cell engineering, the human UC is a very advantageous source of MSCs. Cells from UC are easily accessible, may be processed under GMP conditions, and the isolation of a high number of MSCs can be rapidly achieved in a reproducible manner. Particularly interesting features of UC-MSCs were evidenced in recent years. Due to their youth, UC-derived MSCs exhibit a high proliferation capacity and expansion potential. Thus, compared to other MSC-sources, for UC-derived MSCs no extensive expansion is required to obtain clinical doses, thereby reducing the risk of possible epigenetic damages occurring during the in vitro expansion process. Because one of the challenges of the bioprocesses will be the generation of clinical grade MSCs in disposable reactors, the monitoring of the cultures will be

essential to control cell quality. The development of adequate in situ sensors for the monitoring of the cultures will be of great interest [90]. Furthermore, it has been shown that UC-MSCs can be frozen and thawed efficiently, which makes them suitable for their use in clinical cell banking. The therapeutic use of MSCs will require storage prior to clinical applications. In this regard, it appears worthwhile that UC-cells isolated at birth, may be safely stored and delivered decades later to a patient. Nevertheless, additional studies may be necessary to attest the stability of long term cryopreserved cells.

The clinical potential of MSCs is primary dependent on their differentiation potential. Like BM stromal cells, UC-derived MSCs were demonstrated to be multipotent. Interestingly, their differentiation repertoire does not seem to be restricted to the mesodermal lineages, since the cells could be successfully induced to neurones, liver, and pancreatic cells. A growing body of evidences suggests, however, that UC-MSC populations are rather heterogeneous, harboring a subset of primitive cells. The next generation of studies should focus on the identification and characterization of these sub-populations. In particular, the question of whether the differentiation potential of the isolated populations is dependent on their location in the UC-tissues is of great interest for clinical application. Newly described MSCs markers may be helpful in this regard.

Additionally, first in vitro and in vivo animal studies evidenced immune-privileged and immune-modulatory properties of UC-derived MSCs. Low levels of rejection were observed in all reports of in vivo transplantation experiments and encouraging results in tissue repairs were observed. In particular, supportive function through paracrine effects seems to be involved. The next generation of studies and first clinical trials will clarify whether the benefit of UC-derived MSCs after transplantation experiments relies on supportive effects and/or on differentiation in vivo.

One of the ambitious aims of regenerative medicine is the engineering of tissue in vitro. Few but very promising applications of UC-derived MSCs have been reported in this field. For instance, UC-MSCs are believed to have a high potential in cardiovascular tissue engineering [91]. They grew very well on bio-degradable polymer for the elaboration of cardiovascular constructs [33] and could be used for the construction of human pulmonary conduits [92], for the engineering of biologically active living heart valve leaflets [27], and for the elaboration of living patches with potential for pediatric cardiovascular tissue engineering [93]. The use of newly developed scaffolds, mechanical strain approaches, or 3D bioreactors for tissue generation, which were successfully applied with MSCs from other sources [94], will also be a highly interesting issue.

Considering the very encouraging results obtained in recent years, it may only be a question of time until UC-derived MSCs will be routinely used for clinical and tissue engineering applications.

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Isolation, Characterization, Differentiation, and Application of Adipose-Derived Stem Cells

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Abstract While bone marrow-derived mesenchymal stem cells are known and have been investigated for a long time, mesenchymal stem cells derived from the adipose tissue were identified as such by Zuk et al. in 2001. However, as subcutaneous fat tissue is a rich source which is much more easily accessible than bone marrow and thus can be reached by less invasive procedures, adipose-derived stem cells have moved into the research spotlight over the last 8 years.

Isolation of stromal cell fractions involves centrifugation, digestion, and filtration, resulting in an adherent cell population containing mesenchymal stem cells; these can be subdivided by cell sorting and cultured under common conditions.

They seem to have comparable properties to bone marrow-derived mesenchymal stem cells in their differentiation abilities as well as a favorable angiogenic and anti-inflammatory cytokine secretion profile and therefore have become widely used in tissue engineering and clinical regenerative medicine.

Keywords Adipose-derived stem cells, ASC, Fat harvesting, Isolation protocoll, Stem cell application

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1 Introduction

Despite bone marrow being the primary and best studied source of stem cell populations, multilineage progenitor cells with the potential to differentiate into multiple cell lines have been found in the heart [1, 2], muscle [3, 4], lung [5, 6], intestine [7], kidney [8], liver [9], pancreas [10], skin [11], and even in the brain [12, 13]. These tissue-resident stem cells seem to posses certain repair functions in their native tissue, but also have potential for and might contribute to tissue regeneration processes in regions other than their origin [14, 15].

Due to their many advantages, several recent studies favor adipose-tissue derived mesenchymal stem cells for their study purposes. A multilineage stem cell population that was derived from the stroma of adipose tissue was first described by Zuk et al. in 2001 [16], who used the term “processed lipoaspirate”, as they isolated the cells from an aspirate of a liposuction. Since the lipoaspirate is composed of several cell types, it requires processing by stepwise centrifugation in order to isolate the stem cell fraction.

Fat tissue is composed mainly of mature adipocytes which are aligned in lobules and surrounded by connective tissue. Within the connective tissue, a vascular network allows nutrition and also transportation of endocrine mediators produced by adipose tissue, especially leptin and adiponectin, an insulin-sensitizing hormone [17, 18]. In specific anatomical locations, such as the abdominal wall, the fat layer is divided by a thin facia, so-called Scarpa’s facia, into a superficial and a deeper layer of fat tissue.

After centrifugation of the lipoaspirate, the most voluminous fraction contains adipocytes, which may burst and die during processing. Alongside, another

centrifugation fraction is the so-called "stromal-vascular fraction" (SVF), as the stromal and vascular tissues show nearly identical centrifugation properties. The SVF contains a heterogenous cell population composed of circulating blood cells, fibroblasts, pericytes, endothelial cells, and multipotent stem cells.

Over the past few years, several nomenclatures for these multipotent cells have been used, e.g., processed lipoaspirate, adipose tissue-derived stromal cells, or adipose-derived mesenchymal stem cells, but in this chapter we will use the term "adipose-derived stem cells" (ASC), based on a consensus reached by the Second Annual Meeting of the International Fat Applied Technology Society in Pittsburgh, PA, in 2004, and which is also favored in the literature.

In recent years, research interest in plastic and reconstructive surgery has focused on characterization and tissue engineering approaches of ASC, since these cells are easily and frequently harvested during lipoforming procedures [19–21]. In the following chapter on ASC we would like to define the term "ASC" and its origin. Furthermore, we want to cover the surgical procedures and isolation techniques for ASC from fat tissue. In addition, we want to display the characterization of ASC via surface markers and their differentiation capabilities into different cell lines, e.g., adipocytes, chondrocytes, osteoblasts, myocytes [16], endothelial cells [22], neuron-like cells [23–26], hepatocytes [27, 28], pancreatic cells [29], and hematopoietic supporting cells [30, 31], together with the underlying signal cascades. This will be followed by a short overview of the application of ASC in tissue engineering and in clinical medicine, using the angiogenic, antiapoptotic, hematopoietic, and anti-inflammatory cytokine profile secreted by ASC [31, 32].

2 Isolation of ASC

Due to the easy accessible anatomical location and the abundant existence of subcutaneous adipose tissue, ASC hold the advantage of a simple and above all less invasive harvesting technique. Thus, adipose tissue might be considered as a rich source of stem cells, especially with the increased incidence of obesity in modern populations. In general, adipose tissue can be harvested by liposuction, lipoplasty, or lipectomy procedures. Minimal-invasive procedures such as liposuction or lipoplasty have the advantage of a reduced patient discomfort and lower donor site morbidity compared to lipectomy.

To avoid risks of general anesthesia, small amounts of adipose tissue (100–200 mL) can be obtained under local anesthesia by liposuction procedures. Noteworthy, these "small amounts" are still bigger volumes than those yielded by bone marrow aspiration. In comparison, 1 g of adipose tissue yields approximately 5×10^3 stem cells [33], which is 500-fold greater than the number of MSCs in 1 g of bone marrow [34].

For a complete overview of the harvesting and isolation process, see Fig. 1.

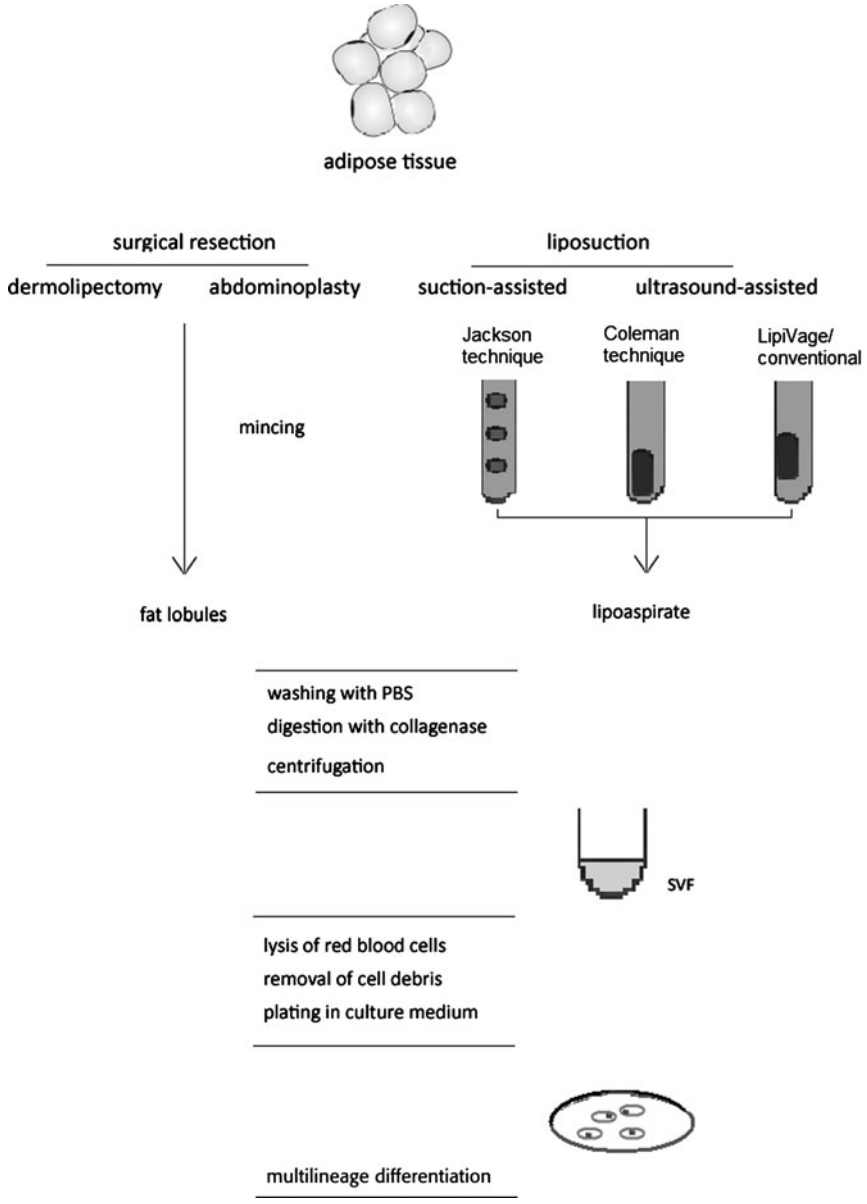

Fig. 1 Flowchart of fat harvesting and ASC isolation process. Note the different cannulas used for the different techniques of fat harvesting

2.1 Influence of Donor Site and Age

Several studies have compared the impact of the isolation procedure via aspiration liposuction, ultrasound-assisted liposuction, or lipectomy on the yield of ASC.

Furthermore, different donor sites such as fat derived from abdominal tissue, hips, or thighs have been compared regarding cell yield, cell viability, and cell differentiating capacity. Interestingly, neither the type of surgical procedure nor the anatomical site of the adipose tissue affects the total number of viable cells that can be obtained from the stromal-vascular cell fraction [33, 34]. However, there is increasing evidence that both the cellular composition and the differentiation capacity display heterogeneity according to the localization of the adipose tissue, at least in the murine model [35].

Since different anatomical localizations of fat tissues have their own metabolic characteristics, such as lipolytic activity, fatty acid composition, and gene expression profile, the source of subcutaneous adipose tissue grafts might influence the long-term characteristics of the fat graft. In rabbits, the osteogenic potential of ASC from the visceral adipose tissue is described to be more effective than those of the subcutaneous adipose tissue [36].

In humans, data from literature are ambivalent; whilst most studies show no difference in the proliferation rate, i.e., the culture doubling time [37–43], there is one study that measured faster proliferation rates in preadipocytes from subcutaneous vs omental adipose tissue (doubling time 4 ± 1 days vs 5 ± 1 days) [44]. This study also found a higher number of endothelial cells in the harvested SVF in agreement with the finding that endothelial cells from adipose tissue were recently described to promote preadipocyte proliferation [45]. Another study describes differences in the frequency of ASC in the adipose tissue from abdominal subcutaneous tissue and from the hip/thigh region with abdominal tissue having superior frequencies, though the absolute cell number was the same [46]. While few studies found attachment and proliferation ratios to be more pronounced in ASC derived from younger donors compared with older donors [40], others found no difference in proliferative capacity concerning the age [41, 42, 44, 45, 47, 48].

Noteworthy, in the study by Zhu et al. [43], though the authors stated ACS from younger donors to be faster proliferating, the difference between young and old donors was slight and not statistically significant.

In addition, the studies mentioned above also examined the differentiation capacity of ASC. Whereas one study examined the adipogenic and osteogenic potential [24], others focused merely on the adipogenic [8, 40, 41, 44, 49] or osteogenic capacity [42, 47, 48, 50]. Interestingly, while some studies found no difference in adipogenesis with regard either to the region [40, 44] or to the age [41], other studies found the potential for adipogenic differentiation elevated in older donors [40, 43].

In contrast, in two studies, differences related to the donor site could be found regarding the adipogenic capacity [8, 49]. Tchkonia et al. compared the differentiation into adipocytes of ASC harvested in abdominal subcutaneous, mesenteric, and omental adipose tissue and observed the highest adipogenic capacity in abdominal subcutaneous adipose tissue, followed by intermediate capacity in mesenteric adipose tissue, and lowest capacity in omental adipose tissue [51]. Hauner and Entenmann found a significantly higher metabolic activity in differentiated

adipocytes derived from ASC, which were harvested from the abdominal subcutaneous adipose tissue compared to ASC obtained from the femoral adipose tissue in obese women [52].

Concerning osteogenesis, the findings were also ambivalent; some studies found no difference in osteogenic capacity [41, 47, 48, 50], others found higher osteogenic differentiation rates in younger donors [43]. The finding that osteogenic capacity is preserved during aging stands in contrast to current clinical experience, where time for fracture healing is enhanced in the elderly or osteoporotic bones compared to the skeleton of younger people. Hence, Khan et al. concede that the donors examined in their study were in their later life (ranging from 57–86) and suffered from osteoarthritis [47].

In summary, general scientific opinion negates any age-dependant effect on ASC proliferation, whereas the influence of age on the differentiation capacity of ASC remains debatable.

2.2 Techniques of Harvesting Fat

Techniques of fat harvesting include direct excision such as dermolipectomy, abdominoplasty, or removal by less or minimal-invasive procedures, such as liposuction. Dermolipectomy comprises the surgical removal of excessive skin and fat tissue from various body locations. The different techniques of abdominoplasty originated from variation in abdominal wall incision for repair of large umbilical hernias in the early 1900s [53]. The modern abdominoplasty as a distinct procedure with umbilical transposition and musculoaponeurotic plication was described by Vernon in the early 1960s and was further refined by others such as Pitanguy, Regnault, and Psillakis [54–59].

After preparation of a whole flap of skin and fat, fat lobules of approximately 0.5–1 cm^3 are separated from the dermo-epidermal layer and minced by repeated cutting with scissors until reaching a paste-like/mushy/pappy appearance. Hereafter, the processing is the same as in liposuction.

Independent of or in combination with an abdominoplasty, the body shape can be modeled by liposuction. Technical aspects of liposuction/lipoplasty include different cannula sizes and shapes, and the use of a wetting tumescent solution, which can drastically reduce blood loss compared to the original dry techniques or ultrasound-assisted devices, which can be advantageous by removing fat tissue from fibrous or scarred areas. Besides ultra-sound-assisted liposuction, which offers very good results concerning body sculpturing but should not be used for harvesting fat due to poor yields of viable cells [60], there are currently four common methods in use for liposuction/lipofilling purposes: conventional liposuction, the method described by Coleman, [61–64], the alternative method developed by Jackson et al. [65, 66], and the LipiVage™ syringe combination.

Whereas liposuction intends to remove fat tissue, devices for lipofilling combine gentle removal of fat with internal processing for reinjection of a viable cell fraction

with the Coleman method being the first and probably best described technique, the Jackson method as a mostly experimentally used technique, and the LipiVage syringe combination as the newest technique.

Conventional liposuction, also called suction-assisted lipectomy, is performed as follows. The patient is brought to a supine position and local, regional, epidural, or general anesthesia is used, depending on the patient's preference or anesthetic risk. A small skin incision with a length of approximately 1 cm is made in the lower abdomen to infiltrate a mixed solution with a blunt Lamis infiltrator. For local anesthesia, the solution contains lactated Ringer's solution with 0.5% lidocaine with 1:200,000 of epinephrine for local hemostasis via vasoconstriction. During epidural or general anesthesia, 1:400,000 epinephrine in Ringer's lactate helps to maintain hemostasis. By using epinephrine, blood loss and contamination of the harvested fat with blood cells is minimized. The solutions are infiltrated at a ratio of 1 cc of solution per cubic centimeter of fat graft to be harvested. An aspiration cannula, 3–4 mm in diameter and 15 or 23 cm in length with a hollow blunt tip, is then connected to a liposuction machine with the negative pressure of the machine being set up at a pressure no greater than 20 cm H_2O (Fig. 1). Fat is harvested through the same incision previously made for infiltration, moving the cannula forward and backwards, disrupting adipose cells mechanically from the surrounding tissue. The adipose aspirate is collected in a sterile bottle; further processing is identical to those of the other methods.

Conventional liposuction is well-tried for the aspiration of fat in the sense of not processing or injecting it thereafter. Many adipocytes are destroyed due to rough mechanical disruption, indicating that other cell types like stromal cells and ASC are also damaged. Thus, the more gentle technique developed by Coleman is widely used for fat transplantation purposes, e.g., body sculpturing or rejuvenation techniques, which is well described and standardized [61–64, 67].

The anesthesia and infiltration technique is the same as described above while, for the harvesting, a different cannula is used. Though it is also 3 mm in diameter and 15 or 23 cm in length, the blunt tip is slightly different. The two distal openings positioned extremely close to the end give the tip a shape reminiscent of a bucket handle (Fig. 1). Also, the harvesting cannula is connected to a 10-cc Luer-Lok syringe instead of using a high-pressure vacuum suction machine. Gently pulling back the plunger of a 10-cc syringe provides a light negative pressure while the cannula is advanced and retracted through the harvest site. Alternative devices that lock the plunger of syringes into place should be avoided, as they can create higher negative pressures that may damage the fragile tissue.

For the technique by Jackson et al. [65, 66], a fine needle apparatus is used together with a 20-cc syringe. The needle for harvest is that used by veterinarians to inject antibiotics into the udders of cows, and it is fitted to a 20-cc syringe. With a diameter of 2 mm and a length of 7 cm, it has a wide bore with a blunt tip and several side holes (Fig. 1). Harvesting is performed as in the other two techniques with forward and backwards motion and intermittent aspiration.

According to recent studies, the technique by Jackson should be favored, as it was superior to the Coleman method in the yield of viable cells in total and ASC in

particular [65], while, in the study by Pu et al. [64], the Coleman method was superior to conventional liposuction although only the yield of adipocytes was measured. Declaring adipocyte viability as a marker for general cellular viability in a fat graft, the chemical effect of epinephrine/lidocaine infiltration remains unnoteworthy, because studies done in the 1990s showed that no chemical damage occurred during liposuction concerning viability of adipocytes [60, 68, 69]. At least, investigations about the influence of infiltration on ASC yield are lacking as yet.

Another promising technique is the LipiVage™-System, quite a new fat harvest, wash, and transfer system which also uses very slight negative pressure, though connected to a vacuum system [49]. While first results revealed superior numbers of viable adipocytes, no data exist for the harvest of ASC at present [49].

In summary, for fat grafting, no matter if intended either for fat transplantation or yield of ASC, conventional liposuction should not be used due to the harsh movements that cause damage to the cells. In contrast, one of the gentle liposuction methods is recommended for harvesting since it has the advantage of a less invasive procedure compared to an abdominoplasty, but regarding the yield of adipocytes, no difference has been found so far between the described techniques [35].

2.3 Isolation of ASC from Fat Grafts

As mentioned before, the isolation processing for the yield of ASC from fat grafts is identical for abdominoplasty- or liposuction-harvested grafts. Most publications use the protocol by Zuk et al. [16], though actually Rodbell pioneered in centrifugation and digestion of fat to identify different fractions in 1966 [70–72]. However, the identification of the SVF was just a secondary product of his work focusing on the metabolism of isolated fat cells, and therefore Zuk et al. are usually referred to as the prime investigators.

After transferring the lipoaspirate or the dissected lobules into a laboratory under sterile conditions following good manufacturing practice (GMP), they are extensively washed in equal volumes of sterile phosphate-buffered saline (PBS). The extracellular matrix (ECM) is digested at 37°C for 30 min with 0.075% collagenase in PBS [73] under permanent shaking. Thereafter, enzyme activity is neutralized with Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS) and centrifuged at 1,200×*g* for 10 min to obtain a high-density pellet of the SVF. The pellet is resuspended in 160 mM NH_4Cl and incubated at room temperature for 10 min to lyse contaminating red blood cells. The SVF was collected by centrifugation, as detailed above, filtered through a 100-μm nylon mesh to remove cellular debris and incubated overnight at 37°C/5% CO_2 at a density of approximately 150,000 cm^{-} in control medium (DMEM containing 10% FBS and 1% antibiotic/antimycotic solution, for example penicillin/streptomycin). Following incubation, the plates were washed extensively with PBS to remove residual nonadherent blood cells. Further cultivation can be done for up to 15 passages before cells become senescent if cells are separated at approximately

60–70% confluence to avoid differentiation due to contact inhibition. A remarkable finding was described by Zhu et al. who passaged ASC cultures up to 25 passages and studied the growth kinetics and differentiation potential [74]. Interestingly, ASC showed several logarithmic growth periods, e.g., the first between the 4th and 7th day of culture, the second between 9th and 10th day, though they allowed cells to reach 90% confluence. While morphology and surface marker did not change between earlier passages and the 25th, multilineage differentiation potential was declined in the 25th passage [75].

Lee et al. observed the greatest numbers of cells obtained from cultures plated at low density [76], so not more than 4×10^3 cells cm^{-2} should be plated per passage. However, before the next passage, a phenotype characterization, for best results with a flow cytometry cell sorter, should follow to cultivate as pure cultures of ASC as possible. Handling these small numbers of cells while culturing, it is noteworthy that cryopreservation is also suitable [77]. ASC appear to be fibroblast-like shaped, expand easily in even FBS-free culture media [78] and can be passaged with Trypsin/ethylene-di-amine-tetra-acetic acid (EDTA) (Fig. 2). The medium should be changed every 2 or 3 days, and passage time is usually about 1 week, depending on the density of plated cells.

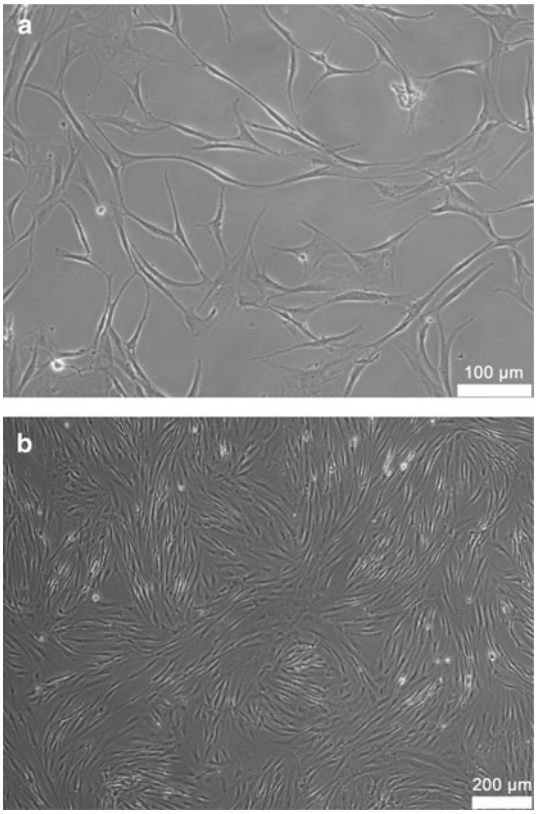

Fig. 2 Isolated ASC in monolayer culture. (**a**) The cells (Passage 0) are seeded to allow cell–cell contacts. Magnification ×100, scale bar represents 100 µm. (**b**) At a higher density the cells begin to form characteristic structures. Magnification ×40, scale bar represents 200 µm

There are slight differences in the isolation process of some authors, mostly concerning the concentration of collagenase or the buffer used [15, 65, 74, 79, 80]. Here, some authors favor longer digestion periods ranging from 30–90 min [15], higher collagenase concentration ranging from 0.05 to 0.15% [15, 77, 79, 80], different collagenases, e.g., collagenase H (1 mg mL^{-1} + 2% bovine serum albumin (BSA)) [65], a different buffer for dissolving the collagenase, e.g., Krebs–Ringer solution (pH 7.4) buffered with 25 mM hydroxyehtyl-piperazine-ethane-sulfonic acid (HEPES) containing 20 mg mL^{-1} BSA [15], or addition of BSA (1–2%) [48, 74]. If dissected lobules instead of lipoaspirates were harvested, Zhu et al. preferred Hank's buffer instead of PBS for washing, while their digestion buffer comprised 0.1% collagenase and 0.25% trypsin in Hank's buffer [74].

Galié et al. compared different centrifugation speeds concerning the yield of ASC and found the best results at 1,200×*g* [81], like in the original protocol. Kim et al. filtered the supernatant through a 70-μm nylon mesh [82–86], while Martinez-Lorenzo et al. preferred the use of first a 100-μm and then a 40-μm nylon mesh [87]; the group around Gonzalez used just a 40-μm nylon mesh [88, 89].

After filtering, Kim et al. used histopaque-1077 as Ficoll gradient in centrifugation [78, 79, 90, 91]. Histopaque-1077 is an inexpensive polysaccharide solution mixed with a radiopaque contrast medium with a density if 1.077 g mL^{-1} which forms a distinct opaque layer. Thus, different fractions of a lipoaspirate can be identified very easily. However, since most other research groups performed isolation of ASC lacking this solution, it may not be necessary for identifying the SVF.

Nagakami et al. used another slightly different protocol [92, 93] developed in 1978 by Björntorp et al. [94], which used a digesting solution containing 0.1 M (HEPES) buffer, 0.12 M NaCl, 0.05 M KCl, 0.001 M $CaCl_2$, 0.005 M glucose, and 1.5% (w/v) BSA with 0.2% (w/v) collagenase at a pH of 7.4 and a temperature of 37°C for 30 min. Filtration was with a 250-μm nylon mesh and after that fat cells were allowed to float to the surface for 15 min. The infranatant was aspired and filtered through a 25-μm nylon mesh and the passing cells were cultured.

Recent studies showed that the growth kinetics of ASC can be influenced by several exogenous supplements. Iwashima et al. displayed that addition of fibroblast growth factor 2 (FGF2) significantly increased proliferation speed of ASC via the FGF-receptor 2 compared with nonsupplemented control medium [95], but FGF-2 also increases chondrogenic differentiation [96].

Proliferation can also be stimulated by exogenous supplementation of sphingosylphosphorylcholine via the activation of c-jun N-terminal kinase (JNK), platelet-derived growth factor (PDGF) via JNK-activation, and oncostatin N via activation of the microtubule-associated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) and the JAK3/STAT1-pathway [97–99].

We would recommend use of the original protocol and modification of some parameters by demand to improve ASC yield. To stimulate proliferation, it may be advisable to add FGF-2 or one of the other mentioned exogenous supplements.

3 Characterization of ASC

Due to the fact that during the isolation process of ASC by stepwise centrifugation and digestion only the SVF can be obtained and this fraction contains a mixture of several cell types, there is a need for further characterization of ASC. In early studies ASC were defined by their ability to differentiate into the adipogenic, osteogenic, and chondrogenic pathway. Nowadays the populations of ASC can be further characterized and sorted by their surface markers using flow cytometry, which is an elegant way to derive pure cell populations from a cell mixture.

Thus, several surface markers have been described to characterize whether a cell is an ASC or not, while some markers have also been found just in subpopulations of ASC.

In the next paragraphs, first specific surface markers are displayed and also gene expression patterns of several proteins typical for ASC are listed. Second, we are going to explain, differentiation pathways and mechanisms of ASC as far as they are revealed.

3.1 Characterization via Surface Marker and Gene Expression

The first publication by Zuk et al. defined the stem cell characteristics of ASC by their ability to differentiate into several mesenchymal cell lineages, such as the adipogenic, osterogenic, chondrogenic, and myogenic lineage [16].

With the following lineage-specific determinants and the matching histological and immunohistochemical assays, the differentiation was proven: Adipogenic differentiation was defined by lipid accumulation (monitored with Oil Red-O stain), osteogenic differentiation by alkaline phosphatase (AP) activity (AP-stain) and calcified matrix production (Von Kossa stain), chondrogenic differentiation by sulfated proteoglycan-rich matrix (Alcian Blue (pH 1.0) stain) and Collagen II synthesis (Collagen II-specific monoclonal antibody), and myogenic differentiation by multinucleation (phase contrast microscopy) and skeletal muscle myosin heavy-chain and MyoD1 expression (Myosin- and MyoD1-specific monoclonal antibodies (Fig. 3).

One year later, the same working group published another study in which they characterized ASC by flow cytometry analysis and made a comparison with BSC [21]. Both populations expressed CD13, CD29, CD44, CD71, CD90, and CD105/SH2 and SH3, which together with SH2 is considered a marker for MSCs [100]. In addition, both ASC and BSC expressed STRO-1, a marker for multilineage progenitors from bone marrow [101]. In contrast, no expression of the hematopoietic lineage markers CD31, CD34, and CD45 as well as absence of CD14, CD16, CD56, CD61, CD62E, and CD104 were observed in either of the cultures. Interestingly, there was a difference between both stem cell lines for the expression of CD49d, which was expressed by ASC but not BSC, and CD106 for which it was vice versa.

A recent study by McIntosh et al. investigated the temporal changes of marker expression on ASC [102]. Hence, markers seem to underlie progression or depression

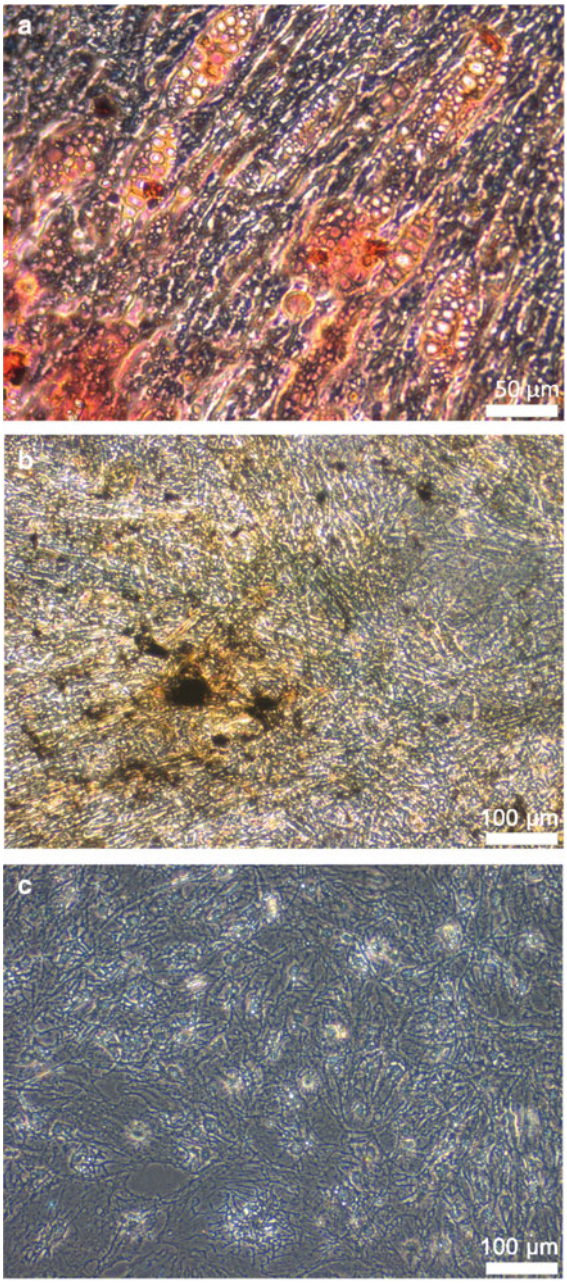

Fig. 3 Histologic staining of differentiated ASC. (**a**) Adipogenic lineage stained with Oil Red-O staining. (**b**) Osteogenic lineage stained with Von Kossa staining. (**c**) Chondrogenic lineage stained with Alcian blue staining. For (**a**) magnification ×200, scale bar represents 50 µm, for (**b**,**c**) magnification ×100, scale bar represents 100 µm

by ongoing passaging, showing a passage-dependent decrease of CD11a, CD13, CD45, CD86, and histocompatibility locus of antigen (HLA)-DR. Instead, CD40, CD54, and HLA-ABC increased during passaging, while CD80 showed growing expression until passage 1 and 2, and then decreased.

In their excellent review, Schäffler and Büchler defined positive and negative markers and genes for ASC with regard to the literature [15]. Thus, ASC are positive for CD9, CD10, CD13, CD29, CD44, CD49d, CD49e, CD54, CD55, CD59, CD73, CD90, CD105, CD106, CD146, CD166, HLA I, Fibronectin, Endomucin, smooth muscle cell-specific alpha actin, Vimentin, and Collagen-I. They are negative for CD11b, CD14, CD19, CD31, CD34, CD45, CD79α, CD80, CD117, CD133, CD144, HLA-DR, c-kit, MyD88, STRO-1, Lin, and HLA II.

Even more extensive is the study by Katz et al. [103], who analyzed the transcriptome of ASC using a microarray technique (Table 1). Though their results demonstrated uniformity in some gene and surface marker expression considering different isolation and cultivation protocols, there seem to be time-dependent changes already seen in short-term culture with decrease of certain surface markers as well as differences in individual gene expression profiles with a 66% consistency in gene expression comparing samples of three persons with gene arrays and seven persons in flow cytometrie [103].

Comparing partly contrary data from different research laboratories, it appears difficult to determine a definitive immunophenotype. Until now, there has been no definitive immunophenotype of ASC as surface markers do change expression during passaging. According to results of different studies, one can agree upon a selected surface maker expression profile as a basic prerequisite in order to define the adipose mesenchymal stem cell: This profile should comprise positivity for mesenchymal stem cell markers such as CD105, CD73, and CD90 as well as lack of the hematopoietic lineage markers c-kit, CD14, CD11b, CD34, CD45, CD79α, CD19, and HLA-DR [78].

Another interesting investigation was carried out by Gonzalez et al. who studied the inhibition of inflammatory and autoimmune responses by undifferentiated ASC [88]. They found not only expression of surface receptors chemokine (C-C motif) receptor 1 (CCR1), CCR2, CCR4, CCR7, CCR9, chemokine (C-X-C motif) receptor 1 (CXCR1), and CXCR5, but also proof for their functionality because ASC migrated in response to chemokine (C-C motif) ligand 5 (CCL5), CCL22, CCL19, CCL25, chemokine (C-X-C motif) ligand 8 (CXCL8), and CXCL13 activation.

This would suggest anti-inflammatory properties as well as immunosuppressive properties mediated by ASC, confirmed by more recent studies which are displayed in the paragraph below regarding immunomodulation by ASC.

4 Differentiation of ASC

The perception of adipose tissue has undergone a radical change over the past 10 years. From a special type of connective tissue with the function to store excess energy as triglycerides, new functions have been assigned to adipose tissue during the past 10 years. Adipose tissue has been described as a real endocrinic organ between neuroendocrine and metabolic signaling [104]. Furthermore, it has been identified as a rich source of multipotent stem cells.

Table 1 Transcription profile of extracellular matrix and angiogenesis-related genes in early-passage, undifferentiated human ASC [103]

Cell adhesion molecules	
Integrins	
Integrin α1	CD49a/VLA-1
Integrin α2	CD49b
Integrin α2b	CD41b
Integrin α3	CD49c
Integrin α4	CD49d
Integrin α5	CD49e/VLA-5
Integrin α6	CD49f/VLA-6
Integrin α7	
Integrin α8	
Integrin α9	
Integrin α11	
Integrin αV	CD51
Integrin αX	CD11c
Integrin β1	CD29
Integrin β2	CD18
Integrin β3	CD61
Integrin β4	
Integrin β5	
Integrin β8	
Cadherins and catenins	
Cadherin 1 type 1	
Cadherin 5	CD144
Catenin β1	
Catenin δ1	
Catenin δ2	
Catenin α1	
Catenin α-like 1	
Other cell adhesion molecules	
GPIV	CD36
H-CAM	CD44
CEACAM-5	CD66e
ELAM-1	CD62e
ICAM-1	CD54
PECAM-1	CD31
VCAM-1	CD106
DCC	
NCAM-1	CD56
Contactin 1	
NRCAM	
Matrix proteins	
Caveolin 1	
Collagen type IV α2	
Collagen type 18 α	
Collagen α1	
Extracellular matrix protein 1	
Fibrinogen B	
Fibronectin 1	
Laminin γ1	
Osteonectin	

(*continued*)

Table 1 (continued)

Osteopontin	
Thrombospondin 1	
Thrombospondin 2	
Thrombospondin 3	
Thrombospondin 4	
Endoglin	CD105
F2, Human prothrombin	
Restin (RSN)	
Vitronectin	
Laminin β1 chain	
MICA	
Proteases	
Matrix metalloproteinases	
Metalloproteinase/METH 1	
Matrix metalloproteinase 2 (MMP2)	
Matrix metalloproteinase 10 (MMP10)	
Membrane-type matrix metalloproteinase 1 (MMP14)	
Matrix metalloproteinase 17 (MMP17)	
Matrix metalloproteinase 26 (MMP26)	
Human stromelysin-3 (MMP11)	
Matrix metalloproteinase 9 (MMP9)	
Matrix metalloproteinase 20 (MMP20)	
Disintegrin-like metalloproteinase	
Matrix metalloproteinase 1 (MMP1)	
Matrix metalloproteinase 3 (MMP3)	
Matrix metalloproteinase 7 (MMP7)	
Matrix metalloproteinase 8 (MMP8)	
Matrix metalloproteinase 12 (MMP12)	
Matrix metalloproteinase 13 (MMP13)	
Matrix metalloproteinase 15 (MMP15)	
Matrix metalloproteinase 16 (MMP16)	
Matrix metalloproteinase 24 (MMP24)	
Other proteases	
Cystatin C	
Cathepsin B	
Cathepsin C	
Heparinase	
Macrophage scavenger receptor 1 (MSR 1)	CD204
Plasminogen activator, urokinase	
Prostaglandin-endoperoxide synthase 1 (COX1)	
Prostaglandin-endoperoxide synthase 2 (COX2)	
Urokinase-type plasminogen activator receptor	
Transmembrane protease serine 4	
Cathepsin L	
Caspase 8	
Meningioma expressed antigen 5 (hyaluronidase)	
Plasminogen activator	
Cathepsin G	
Protease inhibitors	
Plasminogen activator inhibitor, type I	
Plasminogen activator inhibitor, type II	
Protease inhibitor 5	

(*continued*)

Table 1 (continued)

Pigment epithelium derived factor	
Tissue inhibitor of metalloproteinase 1	
Tissue inhibitor of metalloproteinase 2	
Tissue inhibitor of metalloproteinase 3	
Growth factors and receptors	
Ephrin family	
Ephrin-A2	
Ephrin-B2	
Ephrin-A5	
Ephrin-B5	
Fibroblast growth factors and receptors	
Fibroblast growth factor 1 (acidic)	
Fibroblast growth factor 2 (basic)	
Fibroblast growth factor 4	
Fibroblast growth factor 6	
Fibroblast growth factor 8 (keratinocyte growth factor)	
Fibroblast growth factor receptor 1	
Fibroblast growth factor receptor 2	
Fibroblast growth factor receptor 3	
Fibroblast growth factor receptor 4	
Platelet-derived growth factors and receptors	
Platelet-derived growth factor α	
Platelet-derived growth factor-BB	
Platelet-derived growth factor receptor α	CD 140a
Platelet-derived growth factor receptor β	CD140b
Platelet factor 4	
Transforming growth factors and receptors	
Transforming growth factor α	
Transforming growth factor β1	
Transforming growth factor β3	
Transforming growth factor β receptor 1	
Transforming growth factor β receptor 2	
Transforming growth factor β receptor 3	
Transforming growth factor β2	
Vascular endothelial growth factors and receptors	
Vascular endothelial growth factor D	
Placental growth factor	
Vascular endothelial growth factor	
Vascular endothelial growth factor B	
Kinase insert domain receptor	
Tyrosine kinase, endothelial	
Tyrosine kinase with immunoglobulin and EGF homology domains	
Vascular endothelial growth factor C	
Vascular endothelial growth factor receptor	
Other growth factors and receptors	
Angiogenin	
Angiopoietin-1	
Angiopoietin-2	
Angiostatin binding protein 1	
Chromogranin A (parathyroid secretory protein 1, precursor for vasostatin)	
Epidermal growth factor receptor	
Hepatocyte growth factor	

(*continued*)

Table 1 (continued)

Insulin-like growth factor 1
Melanoma growth stimulator activity α
Nitric oxide synthase 3
Endothelial differentiation sphingolipid G-protein-coupled receptor 1
Epidermal growth factor
Cytokines and chemokines
Interferon β1
Interferon γ
Interleukin 8
Interleukin 10
Interleukin 12A
Midkine (neurite growth-promoting factor 2)
Neuropilin
Prolactin
Tumor Necrosis Factor α
Vascular endothelial cell growth inhibitor
Colony stimulating factor 3 (granulocyte)
Interferon α1
Pleiotrophin (heparin binding growth factor 8/neurite growth-promoting factor 1)
Small inducible cytokine A2
Transcription factors
DNA-binding protein inhibitor
Inhibitor of DNA binding 3 (dominant negative helix-loop-helix protein)
Mothers against decapentaplegic, Drosophila homolog 1
V-ets avian erythroblastosis virus E26 oncogene homolog 1
Hypoxia-inducible factor 1 (basic helix-loop-helix transcription factor)
V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2

H-CAM Homing-associated cell adhesion molecule, *CEACAM* Carcinoembryogenic antigen-related cellular adhesion molecule, *ELAM-1* Endothelial leukocyte adhesion molecule1, *ICAM-1* Intercellular adhesion molecule 1, *PECAM-1* Platelet/endothelial cell adhesion molecule 1, *VCAM-1* Vascular cell adhesion molecule 1, *DCC* Deleted in colorectal carcinoma, *NCAM-1* Neural cell adhesion molecule 1, *NRCAM* Neuronal cell adhesion molecule, *MICA* MHC class I chain-related protein A

Although there has been some theoretical discussion about the origin of stem cells as true residents of fat tissue or as migratory mesenchymal stem cells passing through, there is a broad consensus that fat tissue is a rich source of multipotent cells which can be differentiated into adipogenic, osteogenic, and chondrogenic lineages. Myogenic and neurogenic differentiation potential has also been described [105, 106], as well as single reports about hepatic differentiation [27] and differentiation into endothelial cells which is under current debate [107].

4.1 Adipogenic Differentiation

The multipotent stem cells residing in the vascular stroma of adipose tissue give rise to adipocytes in a highly regulated way characterized by uniform steps starting with

an initial commitment, in which cells are determined in the adipogenic lineage without yet expressing markers of terminal differentiation. Although the molecular trigger which converts the multipotent stem cells into preadipocytes has not been identified, it was demonstrated that treatment with bone morphogenic protein 4 (BMP4) induces adipocyte commitment of the multipotent stem cell line C3H10T1/2 via an activation of the Smad signaling pathway by phosphorylation [108]. An identification of the transcription factors controlled by this signaling pathway might help one to understand the factors necessary for adipogenic determination [109]. Recent studies suggest that regulation of the Wnt pathway is important in adipogenesis [110]; further evidence is given by the fact that expression of Dickkopf (Dkk)1 and secreted frizzled-related protein (sFRP) is necessary for differentiation of ASC [111]. Characteristic genes of adipogenic differentiation include peroxisome proliferator-activated receptor-γ (PPARγ), lipoprotein lipase (LPL), glycerol-3-phosphate dehydrogenase (GAPDH), and glucose transporter 4 (Glut4), whereas adipogenic inhibitory genes like preadipocyte factor-1 (Pref-1) are repressed [112].

A high cellular density [113] and a subsequent growth arrest at the G0/G1 boundary are important prerequisites for preadipocyte differentiation [114]. FGF2 enhances adipogenic differentiation in ASC which in turn is [113] stimulated by 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin, and indomethacin [115]. Thiazolidinediones like trogitazone, pioglitazone, and rosiglitazone are considered to be strong inducers of adipogenic differentiation by binding to PPARs [116]. Hong et al. achieved an improvement of adipogenic differentiation in vitro by supplementation with 17-β estradiol [117] consistent with the regulative influence of sexual steroid hormones on adipocyte development [118].

4.2 Osteogenic Differentiation

Since their first description by Urist [119], the osteo-inductive potential of bone morphogenetic proteins (BMPs) has been well studied. The transcriptional activator Runx2/Cbfa1 acts downstream of the BMP signaling pathway on the expression of osteogenic genes including osteopontin (OPN) and Collagen type 1 subtype A1 (COL1A1) [120].

Medium formulas which have been shown to induce osteogenic phenotypes of ASC include supplementation with dexamethasone, β-glycerolphsphate, and 1,25-dihydroxyvitamin D_3.

Mischen et al. examined the osteogenic differentiation capacity under different growth conditions with variable supply of oxygen and nutrients [121]. They demonstrated a dependence of osteogenic differentiation on sufficient availability of glucose and/or oxygen with concentration ranging from physiologically normal to high. From these results the authors concluded that therapeutic use of ASC in an hypoxic environment, e.g., ischemic osseous defects, require supraphysiologic concentration of glucose and glutamine to compensate for the lack of oxygen. Knippenberg et al. combined stimulation by 1,25-dihydroxyvitamin D_3 and fluid shear stress to induce

osteogenic differentiation, demonstrating that the mechanosensitivity in these cells leads to increased expression of marker genes for bone cell development [121]. It has been assumed that mechanical loading increases gene expression of spermidine/spermine N1-acetyltransferase (SSAT), a regulator of polyamine catabolism, which in turn modulates nitric oxide (NO) production and cyclooxygenase 2 (COX2) gene expression, which indicates a bone-cell like response to mechanical stimulation [122].

Several groups induced osteogenic differentiation by genetic manipulation of ASC. Stimulation with osteogenic protein -1 (OP-1) induced osteopontin secretion and the production of mineralized nodules in murine ASC [123]. Dragoo et al. demonstrated that adenoviral transmitted transfection of ASC with BMP-2 led to bone induction comparable to cells treated with recombinant BMP-2 [79]. Rat ASC transduced with human BMP-7 gene differentiated in vitro into osteoblasts, producing osteocalcin and a mineralized matrix [124]. Transduction of ASC with the osteogenic transactivator Runx2 by adenoviral gene delivery induced osteoblastic gene expression [125] equally successfully, which means that the implementation of effectors downstream of BMP are also suited for the induction of osteogenic differentiation in ASC. The idea to integrate tissue engineering approaches into single surgical procedures prompted Helder et al. to investigate the possibility of short term stimulations to induce osteogenic differentiation. In their study, ASC have been stimulated for 15 min with BMP-2 and BMP-7. While BMP-7 induced a more chondrogenic phenotype, a short-term stimulation with BMP-2 resulted in an increase of Runx-2 and osteopontin gene expression in the stimulated cultures after 4 days; gene expression, however, decreased to control values in 14-day-old cultures [126]. So principally short inductions may be effective but other supporting schemes have to be implemented to achieve a stable osteogenic phenotype.

4.3 Chondrogenic Differentiation

The chondrogenic potential of ASC has been described as somewhat impaired compared to bone marrow derived stem cells which might depend on higher expression rates of Integral membrane protein 2A (ITM2A) [127]. Culture medium should contain tissue growth factor β1, ascorbate-2-phosphate, and dexamethasone [128]. The regulative pathways leading to chondrogenic differentiation of ASC are less well characterized than the pathways for adipose and bone differentiation but it has been found that BMP-4, TGF β3, as well as the Smad 1, 2 and 6 are involved. The growth and differentiation factor-5 (GDF5), which is an important factor in chondrogenesis [129], is also able to promote chondrogenic differentiation in ASC transduced with an adenovirus carrying gdf5 [130].

Chondrogenic differentiation depends on a sufficient supply of oxygen and nutrients [131]. A high cellular density is also a prerequisite for chondrogenic differentiation [132]. The working group of M. Longaker achieved chondrogenic differentiation in a 3D micromass culture system [133]. Additional information was provided by Lu et al. based on their findings that ASC culture in collagen II

enhanced chondrogenic gene expression. The authors could correlate this with a downregulation of Rock 2 gene expression and assume that the differences observed result from a more rounded cell shape associated with culture in collagen II gels under a β1 integrin-Rho A/Rock signaling pathway [134]. Controlled release of TGF-β1 from gelatine-chitosan microspheres resulted in enhanced expression of the chondrogenic marker proteins collagen II and aggrecan when compared to controls treated with gelatine microspheres [135].

Chondrogenesis was enhanced by stimulation with FGF-2 [96]. It was reported that extended passaging (P4–P9) resulted in enhanced expression of aggrecan in ASC stimulated with BMP-6 [136] which is a potent inducer of chondrogenic differentiation [91].

5 In Vivo Applications

Since several studies have been carried out concerning the application of bone-marrow derived mesenchymal stem cells (BSC), studies concerning the application of ASC display a rapidly growing field in cell therapy. Because of their differentiation potential and easy-to-obtain location, ASC display at least an equivalent, but probably a superior, alternative. Due to the more comfortable yield and higher cell numbers, they seem to be more practical in use than BSC [33, 34].

As previously reported, ASC secrete a favorable cytokine profile for biological applications [31, 32, 137]. This profile is certainly one reason for the impressive biochemical properties of those cells.

5.1 Cytokine Profile Secreted by ASC

A review of the literature regarding clinical applications of ASC reveals outstanding properties of these cells, mainly caused by secretion of a very favorable mixture of cytokines, which is not only angiogenic, but also immunosuppressive and antioxidative [138].

Rehmann and coworkers analyzed the cytokine profile of ASC in 2004 and found large amounts of vascular endothelial growth factor (VEGF; 1203 ± 254 pg 10^{-6} cells), hepatocyte growth factor (HGF; 12,280 ± 2944 pg 10^{-6} cells), and tissue growth factor-β (TGF-β; 1247 ± 346 pg 10^{-6} cells), but only small amounts of granulocyte/monocyte-colony stimulating factor (GM-CSF; 84 ± 15 pg 10^{-6} cells) or basic fibroblast growth factor (bFGF; 124 ± 13 pg 10^{-6} cells). Strikingly, when cultured in hypoxic medium containing just 1% O_2 instead of 21% O_2, VEGF secretion increased nearly fivefold (from 1203 ± 254 pg 10^{-6} cells to 5980 ± 1066 pg 10^{-6} cells), which is consistent with other studies implying hypoxia as a significant stimulus for angiogenesis at least for ASC [32].

In a study by Kilroy et al., HGF secretion could be induced by treatment of bFGF or epidermal growth factor (EGF) [31]. Additions of 10 ng mL^{-1} of either bFGF or

EGF resulted in a 2- to 20-fold increase of baseline secretion of HGF, while the presence of ascorbat-2-phosphate additionally to bFGF or EGF, respectively, increased HGF secretion even more. Together, ascorbat-2-phosphate and bFGF or EGF amplified HGF secretion to the 2.0- or 6.3-fold of just the growth factor alone.

Interestingly, neither one of those factors alone nor coaddition with ascorbat-2-phosphate enhanced secretion in differentiated adipocytes. In that study, it was also found that secretion of proinflammatory factors can be induced by treatment of ASC with lipopolysaccharide (LPS), probably due the presence of toll-like receptors in ASC [139]. Using enzyme linked immuno-sorbent assay (ELISA), the concentration of different cytokines was analyzed for time dependence.

Thus, exposure to LPS for 24 h increased the secretion levels of proinflammatory cytokines, though they show different temporal secretion levels. While interleukin (IL)-6 and IL-8 exhibited their maximal mean level of 7845 pg mL^{-1} or 6506 pg mL^{-1} ASC-CM respectively, tumor necrosis factor α (TNF α) reached its peak (maximal mean level of 112 pg mL^{-1}) after 8 h of LPS exposition and declined after 24 h. The hematopoietic, but also proinflammatory cytokines macrophage-colony stimulating factor (M-CSF) and GM-CSF were also induced to a peak of 976 and 52 pg mL^{-1}, respectively, after 24 h induction. The levels of the B-cell inductive factor IL-7 and of the proinflammatory cytokine IL-11 were low, but displayed significant induction of LPS after 24 h, reaching maximal mean levels of 3.4 and 12.7 pg mL^{-1}, respectively, while neither IL-1α, IL-1β, nor IL-12 were detectable in the ASC conditioned medium (ASC-CM). While ASC were not as effective as marrow-derived stroma (MdS) in supporting formation of clonogenic myeloid cells (CMC) out of $CD34^{+}CD38^{neg}Lin^{neg}$ cells (64.1 ± 11 CMC out of 100 $CD34^{+}CD38^{neg}Lin^{neg}$ cells for MdS vs 24.7 ± 9 CMC for ASC), they can actually be stated as hematopoietic effective.

In a study by Bhang et al., influence of hypoxia, addition of FGF-2, and additional supplement of FGF-2 in hypoxic conditions were investigated [140].

While hypoxia and exogenous FGF-2 each elevated VEGF, endogenous FGF-2, and hypoxia-inducible factor (HIF)-1α, but not HGF secretion, supplementation of both together led to further increase of VEGF, endogenous FGF-2, HIF-1α, and HGF secretion.

The findings of the cytokine profile ASC were quite similar in a study by Kim et al. which revealed the following cytokine levels in ASC-CM cultivated for 72 h [85]: platelet-derived growth factor (PDGF; 44.41 ± 2.56 pg mL^{-1}), placenta-derived growth factor (PlGF; 37.87 ± 1.69 pg mL^{-1}), bFGF (131.35 ± 30.31 pg mL^{-1}), keratinocyte growth factor (KGF; 86.28 ± 20.33 pg mL^{-1}), TGF-β1 and -β2 (103.33 ± 1.70 and 75.42 ± 95.98 pg mL^{-1}), HGF (670.94 ± 86.92 pg mL^{-1}), and VEGF (809.53 ± 95.98 pg mL^{-1}). In addition, the values of type I collagen and fibronectin were measured, showing interestingly large amounts, containing more than 1000-fold the values of cytokines (921.47 ± 49.65 ng mL for collagen I and 1466.48 ± 460.21 ng mL^{-1} for fibronectin).

In a study by Wei et al. that researched the application of ASC-CM in a mouse model of hypoxic brain injury (see Sect. 4.3 for further details), contents of brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF)-1 were detected, suggesting also neuroprotective properties of ASC [141].

Additionally, angiogenic matrix metalloproteinases (MMP), MMP-3 and MMP-9 in particular, were detected in ASC-CM in higher amounts than BSC conditioned medium [137]. In this study, amounts of MMP-1, MMP-2, tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2, TIMP-3, monocyte-chemoattractant protein (MCP)-1, MCP-2, and granulocyte chemotactic peptide (GCP)-2 were also detectable in ASC-CM, though in low amounts, which could be slightly increased by culturing in endothelial growth medium for microvascular cells (EGM-MV).

Proteomic analysis for antioxidants in ASC-CM revealed the presence of the precursors of insulin-like growth factor-binding protein (IGFBP)-3, IGFBP-4, IGFBP-5, IGFBP-6, IGFBP-7, IL-6, IL-8, latent transforming growth factor beta binding protein (LTBP)-1, LTBP-2, pigment epithelium-derived factor, superoxide dismutase (SOD)-2, SOD-3, and glutathione peroxidase [82].

A study by Kang et al. investigated the mRNA-expression of different immunomodulatory cytokines, revealing a superior expression of immunosuppressive factors [142]. Constitutive expression was measured for TGF-β, IL-6, IL-8, CCL2, CCL5, VEGF, HGF, COX2, TIMP-1, and TIMP-2, but not for IL-4, IL-10, IL-13, IL-17A, Interferon (IFN)-γ, and GM-CSF. Moreover, TNF-α production of leukocytes cocultured with ASC decreased significantly, whereas TGF-β, IL-6 and IFN-γ production significantly increased in ASC when cocultured with leukocytes. Immunomodulatory factors of ASC, such as TGF-β, HGF, prostaglandin E2 (PGE2), and indoleamine-2,3-dioxygenase (IDO) increased significantly in an ASC/leukocyte coculture.

Taken together, the mixture of constitutively or inducibly expressed angiogenic, hematopoietic, and immunomodulatory mediators secreted by ASC suggest a major influence of ASC on other cell types.

Several studies provided evidence for a humoral effect of ASC on other cells by in vitro tests, in which direct cell–cell contacts were avoided by trans-well assays or by addition of ASC-conditioned medium. Further in vitro and in vivo analysis confirmed (see following paragraphs for further details) that no direct contact of ASC to tissue resident cells is required to achieve the desired effect.

5.2 Angiogenesis and Functional Improvement of Ischemic Muscle Tissue

The cytokine profile mentioned above is surely the main reason for the impressive angiogenetic capacity, i.e., the induction of tissue neovascularisation. This capacity is perhaps the most influential property of the ASC when it comes to regeneration because sufficient nutrition and oxygenation is the basic principle for functional tissue regeneration.

Neels et al. studied adipose tissue formation, e.g., a fat pad, from ASC in vivo [143]. For that purpose, they injected 3T3-F442A preadipocytes subcutaneously into nude mice. Surprisingly, they found not just mature adipocytes, but also blood vessel formation in the derived fat pad. To investigate whether these vessels were

derived from the implanted cells or host, they prestained 3T3-F442A cells with fluorescent green cell tracer and afterwards stained the specimen with fluorescent endothelial cell-specific lectin. By this method they could demonstrate that the new vascularization was derived from host cells. New vessel formation by host cells was found in the pads explanted after 1, 2, 3, and 4 weeks, suggesting a common origin of early and late neovascularization. Interestingly, vessel invasion occurred only at specific points in the fat pads, probably due to the fascia surrounding the fat pad. Thus, vascularization displayed as sprouting from larger vessels that were surrounded by nerve bundles that resided just outside the fascia.

Similar findings were observed by Nakagami et al. who applied ASC in an ischemic hindlimb model in vivo first [92].

In preliminary in vitro studies, they found an increase of endothelial cell (EC) viability, migration and tube formation in coculture with ASC, mainly through secretion of VEGF and HGF. The influence of both of these factors was confirmed by antibody blocking, either of one factor alone or of both together. This also revealed synergistic effects as blocking of one factor inhibited EC viability by 25% (VEGF) or 23% (HGF), respectively, and EC migration by 48% (VEGF) or 26% (HGF), respectively, while blocking of both of them returned EC viability and migration to the baseline level.

The in vivo model of hindlimb ischemia was achieved by ligation of the distal potions of the femoral artery and saphenous vein as wells as dissection of the side branches. ASC were harvested from the inguinal adipose tissue of the same mice, and the mice were divided into three groups. In one group, just PBS was injected into the ischemic hindlimb 10 days after ischemia generation, in the second and in the third, each 1×10^6 ASC in endothelial growth medium (EGM) either enriched with or without growth-factors were injected. Then, 2 and 4 weeks after implantation, blood flow was evaluated by laser Doppler imaging, which showed enhanced recovery in the ASC application group compared to the control group and further improvement in the group with application of ASC combined with growth-factor riched EGM.

Histological analysis of the thigh adductor muscle revealed capillary density depending on change in the blood flow level with the capillaries being positive for von Willebrand factor as endothelial marker. Interestingly, the injected ACS did not show any positive immunofluorescence staining for von Willebrand factor. Thus, they stimulated capillary ingrowth without endothelial differentiation of themselves.

In 2006, Moon et al. studied dose and time dependency of ASC injection in the hindlimb ischemia model by ligation of the femoral blood vessels. He assessed blood flow by laser Doppler flowmetry and muscle necrosis by histological and immunohistological analysis [144].

Ischemic hindlimbs in the ASC group showed normal appearance while mice receiving just PBS injection experienced severe ischemic damage with limb contracture and a 60% incidence of autoamputation after 28 days. The blood flow in the ASC treated group was significantly higher than in the PBS-treated control group, actually nearly comparable to the nonischemic control mice. Histological observation showed functional muscle fiber arrangement vs muscle necrosis in the control

group, while immunostaining with von Willebrand-factor antibody also revealed intact blood vessels vs blood vessel necrosis in the control group. These finding were dose-dependent, as ASC injections with cell numbers ranging from 10^5 to 10^6 cells resulted in better clinical and histological outcomes in the higher cell numbers. Surprisingly, the time-dependence of ASC injection after vessel ligation resulted in better endpoints of blood flow in delayed application of ASC 7 days after vessel ligation compared to application of ASC 24 h after the start of ischemia. The authors explained these findings to be related to better cell survival by subsidence of inflammatory responses in the hindlimb. Due to the high cell numbers required for cell transplantation, especially when they should be yielded from small amounts of tissue, a later injection is also favorable to expand ASC before transplantation.

The same workgroup found an essential role of MMP for vessel formation in an additional study in 2007 [145]. Especially MMP-3 and -9 seem to have outstanding importance for the formation as inhibition by GM6001 decreased in vitro endothelial tube formation of ASC. The same effect could be observed by transfection of ASC with silencer RNA for MMP-3 and -9 in vitro, which was confirmed in vivo in a similar mice model. Mice transplanted with MMP-3 or -9 silencer RNA (siRNA) oligonucleotides-transfected ASC showed lower blood flow recovery and higher tissue injury compared to mice transplanted with ASC which were transfected with control oligonucleotides. Comparison of injection of ASC or BSC demonstrated improved recovery from hindlimb ischemia in the ASC group. This finding suggest a role of MMP-dependent angiogenesis as BSC displayed lower MMP expression rates in RT-PCR analysis compared to ASC.

Furthermore, Fang et al. found an important therapeutic role for stromal cell-derived factor 1 (SDF-1), a factor which mobilizes endothelial progenitor cells from the bone marrow. In this study, intraperitoneal injection of a neutralizing anti-SDF-1 antibody in an ischemic hindlimb model resulted in smaller numbers of circulating EPC and less therapeutic efficacy of ASC [146]. A positive correlation between ASC injection and increase of SDF-1 from the ischemic tissue was found, causing higher numbers of circulating EPC. These results suggest SDF-1 being one of the main factors responsible for the neovascularisation of ischemic tissue mediated by ASC implantation. Interestingly, VEGF gene expression was not detected in ischemic tissue, but in ASC, which implicates a complex orchestration of growth factors by implanted ASC.

Hypoxia has recently been shown to increase angiogenic growth factor secretion, e.g., VEGF and HGF, which suggests an essential role for ASC in tissue recovery after injury, usually leaving tissue regions with suboptimal nutrition [140]. The same effect, i.e., local increase of angiogenic growth factors can be achieved by supplementation of FGF-2, which also supported local survival of ASC and neovascularisation in an ischemic hindlimb model.

Functional improvement of ischemic muscle tissue by ASC transfer was also observed in a myocardial infarction model by Wang et al. [147]. ASC were labeled before implantation with superparamagnetic iron oxide (SPIO) and Lenti-GFP-vectors. One week after ligation of the left anterior descending coronary artery (LAD), ASC were implanted and rats were allowed to recover for 4 weeks. Left

ventricular (LV) function and thickness of the myocardial wall were monitored with magnetic resonance (MR) imaging. ASC implanted animals showed significantly higher LV ejection fractions than control groups as well as a thicker LV formation and smaller ischemic size in the infarct area, which was approved by histological analysis. Indeed, SPIO-containing GFP-labeled ASC were found in the infarct rim and infarct core, indicating direct involvement of ASC in infarct remodeling.

The purpose of another study by Schenke-Layland was to investigate the effect of freshly isolated ASC on engraftment, LV-function, and remodeling of myocardial tissue [148].

Myocardial infarction (MI) was created by ligation of the LAD for 45 min, followed by a stabilizing phase of 15 min and then injection of ASC harvested from GFP-expressing rats in the chambers of the ischemic LV of Lewis rats. Functional assessment was revealed by echocardiography (ECG) prior to MI as well as 6 and 12 weeks after MI. ECG-evaluated ejection fractions, stroke volumes, and cardiac outputs were compared to the preinfarction values, which displayed a significantly better LV function of the ASC-treated group vs the saline-treated control group. While no significant engraftment of the infracted area could be found, remodeling was effectively prevented by ASC treatment, demonstrated by histological examination.

In most recent studies, heart failure caused by myocardial infarction was also treated with ASC injection. Recovery of heart function was monitored with MR and ECG [147, 149]. In the study by Wang et al., improved heart function and increased capillary density was observed though only 0.5% of the implanted cells differentiated to cardiomyoblast-like cells (CLC). These findings were supported in a study by Okura et al. in a coronary ligated mouse model in which one group of human ASC were differentiated to CLC prior to transplantation by induction with 0.1% dimethyl sulfoxide for 48 h, while another group of ASC remained undifferentiated. Cell incubation at 20°C for 20 min achieved spontaneous detachment as monolayers, and transplantation of these monolayer-patches was performed directly into the infracted area of the heart. CLC differentiation was confirmed by evidence of the cardiac enzymes alpha-cardiac actin, myosin light chain, and myosin heavy chain. Although patch-transplantation resulted in short-term improvement evaluated by ECG, long-term improvement was only obtained in the CLC-transplanted group. Histological analysis revealed engraftment of CLC to the scarred areas, but not of ASC. CLC also differentiated into human cardiac troponin I-producing cells, and thus resulted in recovery of cardiac function and improvement of the long-term survival.

These findings are supported by results of a study by van der Bogt et al. in which no long-term improvement of heart function could be found after transplantation of neither ASC nor BSC. No ASC were found to be present in the heart after 4 weeks of implantation, which was monitored by in vivo bioluminescence measurement after injection of D-luciferin [150]. While this is in contrast to the results of most other studies, the study mentioned beforehand could explain these findings [149].

In most studies, the main reason for improvement of ischemic myocardium is mediated by neoangiogenesis, derived by growth factors secreted by ASC, while a study by Okura et al. showed that just a very small percentage of undifferentiated ASC stay resident in the infarction area of the heart, in contradiction to the study of van der Bogt et al. [149, 150]. In this context, an in vivo detection of bioluminescence of predifferentiated CLC implanted to the infracted region of the heart could lead to further understanding of repair mechanisms by ASC.

ASC also display homing properties, i.e., trafficking to ischemic tissues if injected intravenously, for which a study by Bailey et al. offers an explanation [151]. In an agent-based computer-simulation, based on 150 rules formulated after an extensive literature review, the adhesion molecule selectin and its cell receptor CD24 was identified as responsible for leukocyte-like "rolling"-properties of ASC, meaning endothelial adhesion-mediated slowing of circulating cells in the blood flow. In this manner, adhesion and extravasation of ASC were also mediated, which could be confirmed in an in vitro model, showing that only a subpopulation of ASC that expressed CD24 slowed down on an immobilized P-selectin coated surface.

With the knowledge of those underlying mechanisms, further studies concerning the guidance of endothelial P-selectin expression in ischemic tissue should be done to uncover the homing processes in vivo.

5.3 Neurological and Skeletal Application of ASC

Interestingly, neuronal recovery is also possible after transplantation of ASC, as the first studies by Kang et al. showed, probably due to the homing to ischemic tissues [152]. Murine ASC were treated with azacytidine to induce neural differentiation, which was confirmed by expression of microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP) as neuron-resident. After transfection with Lac-Z to visualize migration patterns, ASC were transplanted to the lateral ventricle of the brain of a rat model. Though ASC migrated to various parts of the brain, ischemic brain injury induced by middle cerebral artery occlusion (MCAO) increased migration to the injured cortex significantly. While functional deficits showed recovery after transplantation of differentiated ASC, transfection of ASC with the gene of BDNF improved motor recovery of the deficiency.

In addition to Kang's results, Lee and Yoon used human ASC instead of murine stem cells in a similar study design. They monitored the neurological recovery by histological analysis and also performed behavioral tests for which animals were trained 3 days prior to infarction [153]. They found ASC being located in several brain areas but mainly on the borders between infarcted and adjacent (healthy) tissue. Behavioral tests revealed significant improvement of motor function in the ASC transplanted group compared to the control groups which obtained either no or sham injection with PBS after MCAO.

The same migration properties were shown by SPIO labeling of ASC prior to transplantation, monitored via MR imaging [154]. ASC transplantation was

followed by stereotactic imaging directly into areas adjacent to the infarcted tissue, which was verified by postmortem histological analysis 24 h onwards.

Even merely conditioned medium of ASC with the secreted growth factors should beware of long-term tissue loss after hypoxic brain injury, as could be shown in a study by Wei and colleagues [141]. ASC-CM was applied via the jugular vein of neonatal Sprague-Dawley rats that were subjected to brain ischemia 1 h or 24 h after injury. Morphometric analysis was performed 1 week after and behavioral tests and histological analysis 2 months after transplantation. The ASC-CM treated groups displayed less hippocampal and cortical volume loss as well as significantly better results in the behavioral tests. Histological examination also revealed less neuronal loss. In ASC-CM, several neurotrophic factors, IGF-1 and BDNF in particular, were identified as probably responsible for these impressive findings.

ASC transplantation was also beneficial in brain recovery after hemorrhagic stroke induced by intracerebral stereotactic infusion of collagenase and followed by ASC application 24 h afterwards [137]. Cell numbers positively stained for terminal transferase dUTP nick end labeling (TUNEL), myeloperoxidase (MPO), or OX-42, and brain water content were checked 3 days post transplantation as markers for acute brain inflammation, hemispheric atrophy, and perihematomal glial thickness. Additionally, behavioral scores were evaluated 6 weeks afterwards. All markers together with brain atrophy were significantly less in the treatment groups compared to the controls, but strikingly, histological analysis revealed ASC in the perihematomal areas that were stained positive for endothelial markers, i.e., von Willebrand factor and endothelial barrier antigen, but not for neuronal or glial markers, suggesting differentiation into endothelial but not neuronal cells.

In neurotoxic brain damage too, ASC application was successful in recent studies, whether if the cause was glutamate-induction [155] or 3-nitroproprionic acid [156]. In both studies, transplantation of ASC [155] or just injection of BDNF-containing ASC-CM [156] showed significant improvement of neurological functions.

Furthermore, in other locations of the central nervous system, like in spinal cord injury, ASC transplantation led to efficient migration of approximately 35% of the transplanted cells to the site of the injury, followed by significant recovery of motor function in a rat model [157].

Lately, another promising application was the transplantation of a large amount of ASC (approximately 25 million cells) in three cases of multiple sclerosis in a clinical phase I trial [158]. Though MR revealed no significant changes at the lesion sites, subjective and functional improvement appeared in all three cases. Pharmaceutical medication could be drastically reduced while neurological tests had significantly better results than before treatment. These impressive results have been achieved without further treatment.

Besides the neurological, cardial, and muscular applications, ASC have also been used in regeneration purposes of the skeletal system.

Thus, the first application in skeletal tissue engineering using ASC was an extended traumatic calvarial defect, where fibrin glue was used as carrier and

scaffold for the ASC [159]. In this case report, a 7-year-old girl was suffering from a closed, multifragment calvarial fracture after a fall. Conditions were complicated as a bilateral decompressive craniectomy had to be performed because of refractory intracranial hypertension. After secondary replantation of the calvarial fragments with titanium miniplates, progressive and disseminated calvarial bone resorption occurred over several months, probably due to insufficient fixation. Loosening of almost all osteosynthesis plates and chronic infection with accompanying significant bone resorption resulted in an unstable skull. Following resection of the unstable osteosynthesis and scar tissue, an imprint template of the defects was made and two macroporous sheets were manufactured with the imprint. In the sheets, bone was taken from the ilium, milled, and applied to the sheets. In addition, ASC were derived from the subcutaneous adipose tissue, processed, and injected into the sheets. To keep the cells in place, autologous fibrin glue yielded from peripheral blood of the patient 2 days prior was sprayed with a spray adapter into the sheet. A cranial computer-tomography showed marked ossification in the defect areas, depicting that healing processes have been occurred, though it remains questionable how much of the effect was due to conventional bone transplantation and what influence ASC transplantation had.

These findings could be approved by Cowan et al. who used a calvarial defect in a mouse model [160], though they used allogenic cells for regeneration. In their study they also compared ASC with BSC, calvarial-derived osteoblasts, and dura mater-derived cells as well as all of these cell types derived from either juvenile or adult donor mice.

Actually, they found ASC resulting in higher mineralization and metabolization rates and thus bone of higher quality than in the other groups and juvenile cells promoting better outcomes than adult cells. However, bone regeneration occurred in all cell types with BSC rebuilding bone faster than osteoblasts and osteoblasts faster than dura mater-derived cells.

Hattori et al. compared bone formation by allogenic ASC and BSC seeded on β-tricalcium-phosphate (β-TCP) scaffolds that were implanted subcutaneously in the backs of nude mice [75]. While they found no significant morphological differences in scanning electron microcopy, histology, and immunohistology as well as the protein amount of secreted osteocalcin between ASC and BSC, implantation of the β-TCP-scaffold alone gave poor results. Noteworthy, only few cells were stained positive for antimouse osteocalcin while most cells were positive for antihuman osteocalcin. As the authors yielded ASC from humans and purchased human BSC, these results indicate that the new built bone was mostly of stem cell origin.

In contrast to these findings, Follmar et al. prepared bone allografts in a rabbit model by rinsing the bone marrow out, drilling cortical holes in the surface, and cutting the ends off, thus manufacturing 2.5-cm bone tubes [161]. Those tubes were either implanted into rabbits subcutaneously without further modification, filled with fibrin glue, filled with fibrin glue containing undifferentiated ASC, or filled with fibrin glue containing predifferentiated ASC. Six weeks after implantation, tubes were explanted and examined histologically. In the bone tubes without any supplement, a foreign body reaction, i.e., fibrous encapsulation, could be observed.

The fibrin glue tube revealed a mild inflammation reaction, especially at the cortical perforations, indicating that fibrin glue provokes an immune response. A mild inflammation can also be observed in the ASC groups as well as acellular blebs, apparently remnants of dead cells. Since lack of oxygen and nutrient supply can be one of the causes for cell death, the fibrin glue envelope around the ASC might have acted as a barrier instead of being a matrix for binding and storage of growth factors secreted by ASC. This hypothesis might have served as motivation for Lendeckel et al. to spray fibrin glue onto the cell-seeded sheets.

Another study by Peterson et al. found poor bone formation by ASC-seeded collagen-ceramic scaffolds implanted in femoral defects of nude mice comparable to unseeded control scaffolds [162]. Results were assessed by radiography, histology, and mechanical testing. However, when ASC were transfected with human BMP-2-carrying adenoviruses, bone regeneration improved dramatically. In mechanical testing, regenerated bones were inferior to undamaged control femurs.

Reviewing the literature for in vivo implantation of ASC to treat cartilage defects, we found two studies using an experimental rabbit model with intraarticular application of ASC seeded on a carrier matrix, i.e., fibrin glue [90] or alginate [163]. In both studies, a defined cartilage defect was created artificially in the knee joint of the animals using a dermal biopsy punch [90] or a small impact machine [163]. Dragoo et al. applied ASC to these chondral defects in a fibrin glue scaffold while Zhang et al. used calcium-alginate as carrier matrix. Prior to application, Dragoo et al. transfected ASC with Lac-Z gene to determine the fate of the transplanted ASC. Results were evaluated by morphological and histological assessment [163] and further on protein and gene level by Western Blot and PCR [90].

In all experiments, ASC groups showed superior results to those of the control group. Zhang et al. found proper chondral tissue in histological analysis as well as in macroscopic inspection, revealing chondrocyte-like cells, thick matrix, and cartilage-like lacunas after 8 weeks and tissue adjacent to native cartilage after 12 weeks, respectively, in the ASC-treated group. In the study by Dragoo et al., all 12 experimental defects showed complete healing, 10 of 12 scaffolds had seamless annealing to the native cartilage. Aggrecan, a superficial zone protein of cartilage, collagen type II messenger ribonucleic acid, and beta-galactosidase as Lac-Z gene product were identified in all 12 experimental specimens, which exhibited a collagen type II:I ratio similar to that of normal rabbit cartilage. Quantitative histologic analysis that evaluated nature, surface thickness, integrity, bonding, and absence of degenerative changes resulted in an average score of 18.2 of 21 in the experimental group, compared with 10.0 in the controls [90].

5.4 ASC for Enhancement and Acceleration of Wound Healing

In recent years, the potential of ASC for wound healing has also been discovered and is currently further tested for future therapeutic options. In 2005, Rigotti et al.

published a first clinical trial concerning wound healing of chronic radiation wound defects in a small patient collective of 20 patients [164]. Caused by external oncologic radiation therapy, lesions may spontaneously appear after no or minor trauma and may rapidly proceed in size in irradiated tissue with former healthy appearance even years after end of the therapy. The most frequent presentation is radiodermatitis with erythema, desquamation, and edema, which evolves over time in subcutaneous fibrosis and, in most critical cases, toward radionecrosis. There are hypotheses that identify vessel hyperpermeability and altered blood flow causing a chronic ischemic status as reason for the tissue damage [165]. Nevertheless, ultrastructural analysis by Rigotti et al. revealed capillary vessels reduced in number with duplication of the basal membrane and ectatic lumina [164]. Treated patients were suffering on progressive tissue lesions, i.e., grade 3 (several symptoms) and grade 4 (irreversible functional damage) according to the LENT-SOMA scale for classification of late radiation morbidity. Purified lipoaspirates taken from a healthy donor site were administered by repeated computer-assisted injection, and therapy outcomes were assessed as downgrading in the LENT-SOMA scale after 18–33 months.

Except for one patient, a significant increase in the scale could be observed, ranging from complete remission to slight improvement, e.g., downgrading from grade 4 (irreversible functional damage) to grade 3 (several symptoms). The authors hypothesized neoangiogenesis in the ischemic tissues as cause for improvement of tissue structure, affirmed by ultrastructural analysis of biopsies from the treated tissue.

Parker et al. described accelerated wound healing in a murine model of impaired wound healing with diabetic db/db mice by injection of allogenic ASC, resulting in wound closure of 92% after 12 days vs 49% in the controls and complete wound closure in the treatment group 1 week sooner than in the controls [166].

These findings could be confirmed by two studies implemented by Nambu et al. dealing either with impaired wound healing caused by topical application of the antimitogenic mitomycin C or in diabetic db/db mice as well [167, 168], though ASC were seeded on an atelocollagen carrier scaffold.

Mitomycin C inhibits proliferation of various cells, including fibroblasts, keratinocytes, and endothelial cells, most likely through inhibition of DNA, RNA, and/or protein synthesis, and thus is suitable for a model of local full-thickness wound healing disorders. Strikingly, enhancement of granulation tissue formation, i.e., thicker granulation tissue and higher numbers of capillaries, by ASC application was statistically significant in the mitomycin C-treated wounds but not in the control wounds [167].

Using diabetic mice displayed similar results, i.e., epithelization rates of 87.3% in the ASC group vs 57.8% in the control group and more than double numbers of capillaries as well as more than double thickness of the granulation tissue.

A study series by Kim et al. on wound healing of skin defects by local application of ASC demonstrated improved and faster wound healing, prevention of photoaging, and antiwrinkle effect in the ASC-treated groups [82–86]. Kim's first study in 2007 measured the effect of secretory factors on human dermal fibroblasts (HDF) [85]. Enhancement of HDF proliferation and migration was promoted by direct

contact with ASC and also by indirect contact through ASC-CM, i.e., serum-free DMEM/F12-medium in which ASC were cultured for 72 h. Growth factors and ECM secreted by ASC were measured with ELISA as well as collagen I, III, fibronectin, and MMP-1 secretion by HDF following induction by ASC-CM. In addition, the therapeutic effect of ASC application in a collagen gel into 7-mm experimental wounds of nude mice was investigated.

Proliferation and migration was significantly higher by culture with ASC and ASC-CM compared to controls. Interestingly, the secretion of collagen I and fibronectin were much higher than those of growth factors with an amount of 921.47 ± 49.65 ng mL^{-1} for collagen I and 1466.48 ± 460.21 ng mL^{-1} for fibronectin, respectively. HDF-production of collagen I, III, and fibronectin was upregulated by induction with ASC-CM while MMP-1 was downregulated. In vivo wound closure after 7 days was also significantly faster in ASC-treated wounds.

In another study in 2008, Kim et al. found evidence for antioxidant action and thus a protecting function of ASC [82]. Actually, ASC-CM had an antioxidant potential comparable to ascorbic acid, measured by an antioxidant assay kit containing the enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), the macromolecules albumin and ferritin as well as an array of small molecules including ascorbic acid, α-tocopherol, β-carotine, reduced glutathione, uric acid, and bilirubin. Furthermore, proteomic analysis of ASC-CM demonstrated increased SOD- and GPx-activity in HDF cultured in ASC-CM after *tert*-butyl-hydroperoxide (tbOOH)-exposure, which causes dose-dependent oxidative injury, as well as increased caspase-3 activity as indicator for apoptosis following tbOOH-exposure.

For results of proteomic analysis, see Sect. 4.1. Though comparable in their antioxidant activity, ASC-CM exhibited a more potent protective effect on HDF than ascorbic acid, probably by scavenging free radicals. ASC-CM increased SOD-activity 1.37-fold and GPx-activity 2.5-fold while ascorbic acid did not change SOD-activity and increased GPx-activity 1.5-fold. The percentage of apoptotic HDF incubated for 24 h with ASC-CM were 8.4 vs 5.1% in controls, 2.8-fold increase in caspase-3 activity after tbOOH-exposure was reversed by ASC-CM (decrease of 2.1-fold compared to controls).

Another factor secreted by ASC, TGF-β1, is responsible for inhibition of melanin synthesis, related to another study by Kim et al. in 2008 [86].

In 2009, Kim et al. could show the antiwrinkle effect of intradermal injection of ASC [83]. An artificial photo-aging mouse model, induced by defined amounts of UVB-radiation, displayed not only dose-dependent reduction of skin wrinkles, affirmed by an optical scoring scale. Dermal thickness increased after mid-level- and high-level-administration of ASC, i.e., injection of 10^4 or 10^5 ASC (16 and 28%, respectively). In addition, UVB-radiation decreased proliferation of HDF, but this effect was altered by pretreatment of HDF with ASC-CM which showed a protective effect on HDF-proliferation.

ASC could be found in skin biopsies 2 weeks after injection, which also resulted in elevated levels of collagen fibers in the dermis.

In 2009, Kim also reported a case report with a single female patient who received two successive intradermal injections into the periorbital region at

2-week-intervals [84]. Two months after the second injection, she showed improvement of general skin texture and wrinkles as well as a slight increase of dermal thickness (2.054 mm before vs 2.317 mm after treatment).

Strikingly, a most recent study by Kim et al. in 2009 could show that VEGF- and bFGF-secretion of ASC is dependent on the oxygen concentration of the culture medium with preference to hypoxic medium [169]. To confirm these findings, experimental wound healing was observed after application of a collagen gel containing ASC-CM after incubation either in hypoxia (2% O_2, 20% CO_2, and balanced N_2) or in normoxia (20% O_2, 5% CO_2), resulting in faster wound closure in hypoxia-conditioned medium.

These latest findings suggest that ASC play an important role in tissue repair after injury. Capillary damage in the wound bed leads to lack of oxygenation and nutrient supply resulting in a hypoxic environment. Therefore, cells that can promote wound healing under hypoxic conditions are most favored, indicating that MSC in general and ASC in particular may represent the body's own potential source for tissue regeneration.

5.5 Immunomodulatory Effect of ASC

The first study concerning the immunomodulatory effects of ASC was published in 2005 by Puissant et al. [170]; this study as well as the one by Keyser et al. [171] and Kang et al. [142] are mentioned here, though they were in vitro studies, due to direct clinical connection.

Puissant et al. tested the response of activated or nonactivated lymphocytes to ASC and BSC to find out either if stem cells would trigger a lymphocyte reaction or if they have an influence on triggered lymphocytes. They found ASC and BSC to be nonimmunogenic while they appeared to be immunosuppressive to activated lymphocytes. This modulatory effect on lymphocytes was obviously mediated by cytokines as it also appeared in a transwell assay, in which ASC and lymphocytes were separated from each other by a porous membrane. Strikingly, these cytokines seem to be induced by lymphocytes as a kind of secretory response, as only a mild immunosuppressive effect was measured when lymphocytes were incubated with ASC-CM, indicating the need for presence of both cell types together to initiate the immunosuppressive reaction.

These findings were approved by Keyser et al. who compared the reduction of T-cell activation by MSC from different tissues [171]. Either Concavalin A or allogenic T-cells were used to induce activation of another population of T-cells. Noteworthy, suppression of T-cell response was most pronounced in MSC from adipose tissue, suggesting them as salvage therapy for suppression of graft-versus-host disease (GVHD).

In 2007, Yanez et al. performed a methodical study to investigate exactly this purpose [172]. Beside in vitro studies, they also implanted bone marrow from

C57Bl/6 mice to B6D2F1 mice irradiated beforehand and compared ASC-injection 0, 7, and 14 days vs 14, 21, and 28 days post implantation.

The remarkable finding was that the early implanted animals had significantly higher survival rates due to GVHD than the later implanted animals.

In contrast to the just mild suppression of ASC-CM alone, Kang et al. found the supernatant of ASC-CM derived from beagle dogs culture to suppress leukocyte proliferation that was stimulated before with Concovalin A, pokeweed mitogen, and LPS [142]. For more detailed consideration of the immunomodulatory cytokine profile of these canine ASC, see the paragraph above concerning cytokine profiles of ASC.

In 2004, LeBlanc et al. reported a case of severe therapy-resistant GVHD of the gut and the liver, which improved rapidly after injection of ASC, which displayed the first use of ASC in clinical medicine [173].

From 2006 on, Fang et al. published a series of studies dealing with the clinical application of ASC for the suppression of GVHD [46, 174–178]. In their study from 2006, they reported a case of a patient who suffered from severe GVHD after stem cell transplantation, which also manifested mainly in the gut and the liver, proven by coloscopy and elevated liver enzymes [178].

After ASC-transplantation from an allogenic donor, symptoms vanished in a few days, indicating immunosuppression.

Another case report referred to a woman with an acute hepatic GVHD caused by hematopoietic stem cell transplantation, who failed conventional immunosuppressive therapy, e.g., cyclosporine and prednisone [177]. Thus, she was treated with tacrolimus, which was discontinued because of deterioration of renal function. As a salvage therapy, she was than treated with ASC, which resulted in rapid and complete resolution of hepatic GVHD as well as renal toxicity.

Similar finding were published, when two pediatric patients suffered from Philadelphia chromosome-positive acute lymphatic leukemia (ALL) or acute myeloic leukemia (AML), respectively [176]. The child with ALL displayed a gastrointestinal manifestation with severe, partly bloody diarrhea, significant loss of weight, and deterioration of general condition 89 days after hematopoietic cord blood transplantation although adequate pharmaceutical immunosuppression was administered.

AML in the other patient was treated with peripheral blood stem cell transplantation and an adequate pharmaceutical therapy regime as well. After 62 days, a strong involvement of the liver in GVHD was observed by elevation of the liver enzymes.

Both patients showed rapid remission of symptoms after injection of allogenic ASC without side effects, which lasted at least for the follow-up of 374 days or 2 years after ASC-injection, respectively.

Comparable success was noticed in six patients in another study suffering from steroid-refractory GVHD after hematopoietic stem cell transplantation because of leukemia [175]. Here different regimes of immunosuppressive therapy were also followed to protect the patients from GVHD, but their condition deteriorated.

Following ASC transplantation, four of the patients kept remission-free for the duration of the follow-up, which ranged between 18 and 90 months. Two of the patients died – one with no obvious response to AMC transplantation of multi-organ failure and one of a relapse of leukemia. However, the patient mentioned finally showed a good response to ASC-therapy while the relapse was 16 month after the ASC-infusion; thus the proven response rate can be stated as five of six patients.

The two studies published lately were case reports of the treatment of either refractory pure red cell aplasia (PRCA) after major ABO-incompatible stem cell transfusion or refractory chronic autoimmune thrombocytopenic purpura [46, 174].

PRCA occurred in two patients in the first mentioned study due to donor-recipient incompatibility, which here was HLA- but not ABO-matched. Although a conditioning therapy with Busulfan and cyclophosphamide was performed in both cases, the clinical appearance of PRCA, i.e., principally reticulocytopenia and thus erythrocytopenia, could not be avoided. Both patients needed a red blood cell transfusion weekly with no beneficial effect from erythropoietin administration.

In the second case, one patient was diagnosed with autoimmune thrombocytopenic purpura, a disorder wherein autoantibodies are directed against platelet surface glycoprotein (GPs), usually GPIIb/IIIa or GPIb/IX. Hereby, splenic platelet destruction is induced and platelet production inhibited, leading to lack of platelets and extended clotting time.

As in the previously described cases, ASC transplantation led to remission, which is continuing up to the publishing date.

In four studies carried out by the work-group around Gonzalez, ASC were also shown to suppress inflammative diseases [88, 89, 179, 180]. Two studies concerned the treatment of inflammative bowel diseases, induced by tri-nitro-benzene sulfonic acid, and sepsis, the two other studies concerned the treatment of an experimental arthritis, induced by immunization against collagen. All of the studies were performed in vitro as well as in vivo in a murine model.

The authors' interesting thesis is that ASC intervene in the regulation of Th1-cells, the key effectors of autoimmune disorders like Crohn's disease or rheumatoid arthritis, by influencing regulatory T-cells which can be considered as influential mediators of regulating the inflammatory response.

Actually, they found downregulation of both Th1-driven autoimmune and inflammatory responses, resulting in amelioration of the clinical and histopathological severity, abrogating body weight loss, diarrhea, and overall survival in the colitis/sepsis studies and joint inflammation in the arthritis studies.

These findings were mediated by suppression of Th1-cells as wells as augmentation of IL-10-producing T-cells, a cytokine responsible for inhibition of T-cell-response.

Also, ASC impaired Th1 cell expansion by direct cell-to-cell contacts and, additionally induced a population of CD4(+)CD25(+)FoxP3(+) regulatory T cells with suppressive capacity on Th1 effector responses in vitro and in vivo.

Taking these findings together, ASC have a great immunomodulatory influence as they are effective suppressors of immune system responses, in particular T cell responses which mediate GVHD as well as autoimmune inflammatory reactions. In the mentioned pioneering clinical studies, they acted as salvage therapy for cases that were refractory to conventional therapy.

Therefore, they may play a bigger role in future directions of immunologic and hematologic therapy as well as in transplantation medicine due to their properties with seemingly lack of side effects.

5.6 Other Purposes in Current In Vivo Application

A quite interesting clinical application for ASC was published by Garcia-Olmo et al. in 2003 describing a case report, in which they used autologous ASC in a fistula in Crohn's disease [181]. Beside the characteristic diarrhea caused by transmural inflammation of the intestine wall, enterocutaneous, rectovaginal, and perianal fistulas display a frequent and often stigmatizing complication of Crohn's disease with an incidence ranging between 17 and 50%, depending on the source. In particular, secreting processes, e.g., vaginal flatus or fetal incontinence in rectovaginal fistulas or secretion of intestinal content through enterocutaneous fistulas, are extremely unpleasant for the patient.

In the reported case, the patient had a history of disease for 11 years prior to the study and had already received several therapeutic trials including surgical treatment, which resulted in remission.

Garcia-Olmo and coworker harvested the SVF via liposuction from subcutaneous fat, processed it, and injected purified ASC superficial into the wall of the fistula. A vaginal flap covered the resected posterior vaginal wall while fibrin glue sealed the perineal hole. After 1 week, the fistula had completely closed, demonstrating epithelization. Due to this, a clinical phase I study was initiated including five patients suffering from Crohn's fistulas that were also treated at least twice medically and surgically [182]. Here, in 75% of the cases, a closure and epithelization of the fistula, stated as healing, occurred. Nevertheless, it is worth mentioning that they found no relationship between the numbers of cells injected and the success of the procedure as well as between the age or gender of the patient and the success.

Two other studies followed, the first being a clinical phase II trial including 35 patients suffering from complex perianal fistulas, and other than Crohn's fistulas, which resulted as well in a healing in 71% of the patients [183]. In the second, the effectiveness of injection of either the SVF or purified and expanded ASC was tested with much better results for the expanded ASC, i.e., healing in 75% of the fistulas vs 25% of the fistulas with the SVF. Obviously, this is due to the content of just 2–5% ASC in the SVF without processing [184].

The same work-group around Garcia-Olmo also used a similar technique for successful treatment of a tracheomediastinal fistula [185]. The patient had

received treatment with Nd-YAG laser resulting in progressive necrosis which led to formation of a fistula with a diameter of 10 mm. Within 3 h of general anesthesia, a lipoaspirate was harvested, centrifuged, purified, and resuspended in fibrin glue followed by a bronchoscopy in which the fibrin glue with the ASC was injected into the cavity of the fistula. General examination concerning clinical symptoms was performed weekly for the first month; flexible bronchoscopy was done every 3 months, which revealed lack of symptoms as well as mayor reepithelization and complete closure of the fistula, leaving only a small depression in the tracheal wall.

Another application already used in clinical medicine is the so-called cell-assisted lipotransfer by the work-group around Yoshimura [186–188], though the first description of implantation of ASC for soft tissue augmentation was by Cho et al. [139]. Either in ASC pretreated in conditioned medium or undifferentiated ASC were implanted subcutaneously in athymic mice, each group supplemented with or without bFGF. Six weeks after implantation, they found not just newly formed adipose tissue but also neovascularisation of this tissue with both predifferentiation and bFGF enhanced these effects.

These finding were approved by Matsumoto et al. who used merely centrifugation to yield human SVF, which was mixed 1:1 with aspirated fat to obtain fat with a high ASC concentration (cell-assisted lipotransfer; CAL), labeled with CM.DiI and transplanted it also subcutaneously to severe combined immune deficiency mice [186]. The CAL fat survival was better than the pure fat controls, resulting in 35% larger specimen derived after transplantation, with SVF-cells stained DiI-positive found between adipocytes and connective tissue. Some of these cells were also positive for von Willebrand factor, suggesting endothelial differentiation.

CAL transplantation in Green fluorescent protein rats also showed neoangiogenesis by SVF-cells in the acute phase of transplantation, indicating CAL as promising tool for long-time survival of fat transplantation.

Actually, this work-group performed two clinical trials, one dealing with facial lipoatrophy and one with breast augmentation, which could prove the success of CAL [187, 188].

In the lipoatrophy treatment study, six patients received autologous SVF from the abdomen which resulted in an average volumetric augmentation between 60 and 80% in the 10 month follow-up examination [188]. As facial lipoatrophy is very stigmatizing because patients can hardly hide it, those improvements display a satisfying result, though refinements should always be aimed for.

The second study examined breast augmentation by CAL in 40 cases. Although, the technique displayed long-term survival and safety, two cases with small cyst formation and microcalcification were detected by mammography at 24 months [187]. Less fat atrophy was observed and the augmented breasts looked more natural than those with artificial implants, which also generally appeared to be harder than the CAL-augmented ones.

Although those findings are impressive, they still raise the question whether CAL is really superior to (pure) fat transfer by methods of proven quality and by an

experienced surgeon. With a careful, gentle, and tissue-conserving technique during liposuction, comparable and repeatable results can usually be achieved, as was reviewed recently in the literature [67, 189].

6 Tissue Engineering Applications

As a general definition, tissue engineering combines the principles of bioengineering, biomaterial engineering, and cell transplantations to generate bio-artificial tissues and organs, thus stimulating the self-regeneration of damaged tissues after the in vivo transfer. Artificial ECM is provided to allow for cell proliferation and differentiation in a three-dimensional cell scaffold. Generally, two classes of biomaterials are used for tissue engineering purposes – naturally derived and synthetic materials; the decision usually depends on the physiological and mechanical requirements as well as the biodegradability.

6.1 Adipose Tissue

In an early approach, Patrick et al. seeded preadipocytes on poly DL-lactic-*co*-glycolic acid (PLGA) scaffolds where they differentiated into mature adipocytes, although they did not reach the size of natural mature adipocytes isolated from epididymal adipose tissue [190].

Adipogenic differentiated rat ASC were seeded on polyglycolic acid (PGA) fibers and implanted subcutaneously onto the heads of rats. Intracellular lipid vacuoles could be demonstrated histologically in the grafts explanted after 4 and 8 weeks [191].

In the study of von Heimburg et al., human preadipocytes were seeded on different biodegradable carriers like HYAFF 11 sponge and collagen sponges. The HYAFF 11 sponges turned out to have a higher cell density than the collagen matrices used [192]. Further modifications including pore size and coating with glycosaminoglycan hyaluronic acid improved cell penetration and vascularization in an in vivo approach [193]. Collagen sponges were also used by Huss et al. for coculture assay with human mammary epithelial cells and preadipocytes. They found lipid-containing cells clustered around ductal structures [194].

Gelatin sponges seeded with human ASC have soft tissue-like mechanical properties which is generally preferable compared to more rigid structures [195]. Hyaluronic acid (HA)-based materials have been used in a variety of tissue engineering purposes and clinical applications [196, 197]. An HA-based scaffold has also been used as a scaffold for adipocyte precursor cells. Full maturation into adipocytes was achieved with an even cell distribution [198].

In a unique approach, in 2008 Vallée et al. [199] completely renounced the substitution of exogenous matrix materials, resulting in three-dimensional cell sheets of adipogenic stimulated cells.

6.2 Bone Tissue

Three dimensional culture has a favorable effect on expression of osteogenic marker genes compared to monolayer culture when ASC are cultured in osteogenic medium [200]. The use of stromal vascular fractions of adipose tissue has been considered advantageous since it enables direct three-dimensional seeding without two-dimensional culture steps for cell expansion [201]. ASC seeded on biomaterials of clinical relevance in the treatment of bone defects like hydroxyapatite, cancellous human bone fragments, deproteinized bovine bone granules, and titanium produce more calcified matrix than cells in monolayers [202].

After 2 weeks of differentiation in porous PLGA foams, mineralized nodular structures were shown [203]. In their rat transplantation model, Lee et al. demonstrated that predifferentiated grafts did not show bone formation but stained positively for osteocalcin after 4 weeks [191]. After 8 weeks, osteocalcin expression was still detectable and, additionally, bone formation was shown in histological analysis.

In their study with electrospun composite scaffolds consisting of beta-tricalcium phosphate (TCP) crystals and poly L-lactic acid (PLA), McCullen et al. provided another indication that matrix properties are important in the differentiation process as alkaline phosphatase activity and mineralization were shown to depend on fiber diameter and TCP content [204].

Shen et al. used a three-dimensional sintered microsphere matrix of poly(lactide-*co*-glycolide) and differentiated using recombinant GDF-5 as an alternative matrix material for bone tissue engineering [205].

Combinations of ECM components are often used to enhance the cell adhesion on hydrophobic synthetic polymers [206].

Beside the administration of modifying substances like growth factors or the transduction with genes of the osteogenic signaling pathway, an optimized differentiation of the ASC is largely influenced by the used scaffold material and stimulation exerted by mechanical forces. Honeycomb-shaped atellocollagen sponges seeded with ASC were successfully used for bone formation in 3D cultures in vitro and in vivo [207]. Osteoinductive materials like β-TCP have been shown to induce osteogenic differentiation [208]. In a comparative study between akermanite ceramics and β-TCP an even higher osteoinductivity was found for the akermanite ceramics [209]. Peterson et al. demonstrated in a combined approach that BMP-2 transfected ASC together with collagen-ceramic carriers resulted in healing of a critical size femoral defect when applied to nude mice [162].

In their approach dealing with the induction of osteogenesis by mechanical forces, van Griensven et al. showed that bone tissue constructs were achieved by seeding adult stem cells derived from bone marrow and adipose tissue seeded on Sponceram matrices in a rotating bed bioreactor [210].

In a combined assay with human ASC seeded on a composite BMP-2 loaded PLGA/hydoxyapatite scaffold were implanted subcutaneously on the backs of

athymic mice without any predifferentiation. After explantation, expression of human-specific osteoblastic genes was found [211].

6.3 Cartilage Tissue

PLGA is a scaffold commonly used in cartilage tissue engineering based on differentiated ASC [212–214]. In their study, Jin et al. combined cellular tranduction with human TGF β2 and cultivation on a three-dimensional PLGA/alginate compound to produce cartilage formation efficiently in vitro and in vivo [215]. While copolymers of PLGA is a common substrate in cartilage tissue engineering due to advantageous mechanical properties, successful cell seeding is often hampered by its hydrophobicity [216]; the authors circumvented this problem by choosing aliginate as a cell carrier.

In another approach ASC were encapsulated in aliginate microbeads together with a chimeric RGD-protein. The parameters analyzed included chondrogenic differentiation after TGFβ3 dependent induction. An increased expression of chondrogenic genes was observed which dependent on a β1 integrin mediated signaling [217].

Cheng et al. worked without stimulation with exogenous growth factors when they seeded ASC on scaffolds derived from porcine articular cartilage. After a cultivation period between 4 and 6 weeks, expression of chondrogenic genes was observed [218]. These finding stress the importance of three-dimensional structures in the chondrogenic differentiation for which there is a broad consensus in the published literature [132–134, 217, 218]. Also mechanical forces like hydrostatic pressure have been found to be important for chondrogenesis and have been implied by Ogawa et al. in their study on ASC seeded in three-dimensional collagen matrices. Cyclic hydrostatic pressure conditions caused an increase in chondrogenic gene expression in TGFβ1 stimulated cultures.

Another possibility for cartilage tissue engineering was described by Hildner et al. when they demonstrated that cocultivation of human articular chondrocytes together with ASC resulted in increased collagen type IX expression, indicating a long-term stability of cartilage [219].

7 Summary

Stem cells derived from adipose tissue represent an enormous potential for medical therapeutic applications. Their versatile properties range from promotion of cell proliferation, differentiation, and migration over immunomodulatory functions, e.g., in GVHD and regeneration to facilitate tissue repair and neovascularization in injury and ischemia. A major advantage for clinical application of ASC is based upon easy harvesting techniques with sufficient yields, vast availability, and low donor site morbidity compared to stem cells obtained from bone marrow or

peripheral blood. The fast expanding field of basic and clinical research in ASC biology, pathophysiology, and their potential therapeutic applications promises further exciting discoveries in the near future.

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Induced Pluripotent Stem Cells: Characteristics and Perspectives

Tobias Cantz and Ulrich Martin

Abstract The induction of pluripotency in somatic cells is widely considered as a major breakthrough in regenerative medicine, because this approach provides the basis for individualized stem cell-based therapies. Moreover, with respect to cell transplantation and tissue engineering, expertise from bioengineering to transplantation medicine is now meeting basic research of stem cell biology.

In this chapter, we discuss techniques, potential and possible risks of induced pluripotent stem (iPS) cells in the light of needs for patient-derived pluripotent stem cells. To this end, we compare these cells with other sources of pluripotent cells and discuss the first encouraging results of iPS cells in pharmacological research, disease modeling and cell transplantation, providing fascinating perspectives for future developments in biotechnology and regenerative medicine.

Keywords Cell transplantation, Differentiation, Induced pluripotent stem cells (iPS cells), Reprogramming, Tissue engineering

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1 Introduction

The isolation and characterization of embryonic stem (ES) cells from mouse blastocysts by Evans and Kaufman in the early 1980s [1] represents a hallmark in stem cell research. Widespread belief was maintained that isolation of ES cells was only possible from certain mouse inbred strains, and isolation of ES cells from other species may not be possible at all due to lack of comparable inbred strains in other species. Just 15 years later, Thomson et al. contradicted this hypothesis by establishing nonhuman primate (NHP) ES cell cultures from rhesus monkey (*Macaca mulatta*) [2], common marmoset (*Callithrix jacchus*) [3], and finally ES cells from humans [4]. Although the ultimate proof of pluripotency by generation of chimeric animals is still pending in these animals, and due to ethical reasons almost impossible in humans, primate ES cells are now generally considered pluripotent based on their ability to form teratomas and to differentiate *in vitro* into cells of all three germ layers. Even redifferentiation into trophoectoderm has been demonstrated [5]. Despite their unlimited potential for differentiation and expansion, the use of human ES cells in research, pharmascreening and cellular therapies is ethically controversial due to their isolation from human embryos and the unavailability of patient-specific cells.

Consequently, a lot of effort was invested into research aiming for generating pluripotent human cells from other sources than preimplantation embryos, which finally led to the induction of pluripotency in "terminally differentiated" cells as demonstrated by Shinya Yamanaka in his groundbreaking Cell paper. This particular study and his subsequent work were a major stimulus to stem cell research, because the two major obstacles to clinical application associated with ES cells were overcome – destruction of human embryos and allogeneic immune rejection [6–8].

2 Needs for Patient-Derived Expandable Cell Sources

In regenerative medicine, several concepts focus on individualized therapies which take advantage of cell-based tissue repair or tissue engineering applications. The use of patient-derived cells will circumvent immunologic issues like rejection of the transplants, but is limited by the availability of suitable (tissue-specific) stem cells

and, in the case of genetic mutations, by gene correction strategies which can be applied to the patients' cells. Moreover, patient-derived cells, which mimic the diseased phenotype, may allow the *ex vivo* exploration of new therapeutic approaches. However, besides the hematopoietic system hardly any other organ is as well understood and, therefore, little is known about progenitor cell types within the cellular hierarchy during organ development which can be expanded *in vitro* for future applications.

For instance, the liver is an ideal target organ for cell-based therapy as demonstrated by the application of hepatocyte transplantation in a number of patients with hereditary metabolic liver disease and acute liver failure [9–13]. In these first clinical studies, hepatocyte transplantation has been considered either as a full treatment option, or in more severe situations, as a bridge to transplantation [14]. In some patients, transplanted hepatocytes are able to engraft, repopulate the liver, and restore the deficient hepatic function for up to 18 months post-transplantation [15, 16] and, meanwhile, more than 20 such patients have been reported in recent years [17]. However, hepatocytes prepared from donor organs can only be provided for a small number of patients and other cell sources are urgently needed. Another example is the engineering of bioartificial cardiac muscle which may allow replacement of infarcted heart tissue. Cardiac tissue engineering is hampered by the fact that adult cardiomyocytes (CMs) have almost no potential for proliferation [18]. In conclusion, for the majority of tissue types, including liver and heart, the lack of suitable cell sources represents one of the major hurdles to be overcome prior to clinical application of novel regenerative therapies.

With respect to adult stem cell sources, recent research suggests strong limitations of adult cell sources with regard to differentiation and expansion potential (see for instance [19–23]), despite a variety of earlier reports suggesting a virtually unlimited plasticity. Consequently, different adult stem cells appear to be useful for therapeutic regeneration of those tissue types, which show a high natural capacity for regeneration, for example, bone or skin. In case of tissue and organs with rather limited natural regeneration potential, for instance the heart, it is still controversial whether adult stem and progenitor cells can prevent loss of function or reconstruction of injured tissue [20–23]. Furthermore, although not proven to the extend, there is a general impression that in older (and diseased) patients, there are less stem and progenitor cells of superior function than in younger donors, which might be due to telomere dysfunctions in aged or stressed cells [24].

In contrast to adult stem cells, pluripotent stem cells, such as ES cells, are characterized by their unlimited potential to grow *in vitro* and to develop into virtually any cell type. As outlined above, pluripotent cells can be isolated from early embryos by collecting blastomeres or by isolating the inner cell mass of blastocysts and subsequent cultivation in appropriate cell culture conditions. Interestingly, these conditions differ distinctly between various mammalian species and to date we are still not able to derive true ES cells from species other than mice [1], NHPs [2, 3], humans [4], and rats [25]. However, various issues need to be considered with respect to application of human ES cells for clinical therapies. Besides strong ethical concerns on destructive use of human embryos, the major limitation for clinical use may be an

immunologic rejection of allogeneic ES cell-derived grafts, which accounts for recent efforts to explore patient-derived pluripotent stem cells.

Recently, it has been demonstrated that pluripotent stem cells can also be derived from embryonic/fetal and adult germ cells. Accordingly, in males, testis-derived cells could serve as an alternative source for autologous pluripotent stem cells [26–31]. In females, pluripotent (embryonic) stem cells can be generated by parthenogenetic activation of oocytes, as demonstrated in mice and NHPs [32]. More recently, mouse parthenogenetic pluripotent stem cell lines were thoroughly described by Kitai Kim [33]. Interestingly, the human stem cell line which was reported by the Korean scientist Woo-Suk Hwang as somatic nuclear transfer (SCNT)-derived cell line was actually a pluripotent stem cell line which has emerged after parthenogenetic activation of an oocyte [33]. The latter two are both germ line-derived pluripotent stem cells, in theory, could be derived from patients, but are not very likely to become an easily applicable cell source for regenerative medicine due to the invasiveness during their isolation procedure. Finally, pluripotent stem cells can be generated through artificial reprogramming of somatic cells, as described in detail below.

3 Induction of Pluripotency and Reprogramming

Using the technique of SCNT, pioneered by John Gurdon [34], the birth of the sheep Dolly in 1996 was the ultimate proof that mammalian cells can be reprogrammed establishing a fully totipotent state. Hereby a somatic nucleus is introduced into an enucleated oocyte arrested at metaphase II stage. These entities are considered to share the same developmental potential with fertilized eggs and can give rise to viable offspring [35]. Other concepts of nuclear reprogramming include the use of ES cells' protein extracts [36], which are able to reprogramme nuclei of fibroblasts into pluripotent cells, or the use of ES cells in fusion approaches resulting in heterokaryons of ES cell and somatic cell origin, whereas the somatic nucleus gains a pluripotency-related gene expression profile [37–39]. Cells generated by these two latter approaches are considered to be pluripotent but not totipotent, mainly because these cells were generated using ES cells.

In 2006, Shinya Yamanaka presented a new concept of reprogramming using retroviral expression of key transcription factors which invalidate with the original transcriptional network of the somatic cells [7]. This pioneering work has been further refined and adopted to the generation of human pluripotent stem cells in recent years [6, 8], and has shown great promise in regenerative medicine (Fig. 1). Even if these iPS cells are considered to share most – if not all – of their molecular characteristics with ES cells, we are still far from providing a concise concept of how reprogramming using the Yamanaka factors (Oct4, Sox2, Klf4, c-Myc) or the Thomson factors (Oct4, Sox2, Nanog, Lin28) works and how this approach can be explained describing the molecular mechanisms.

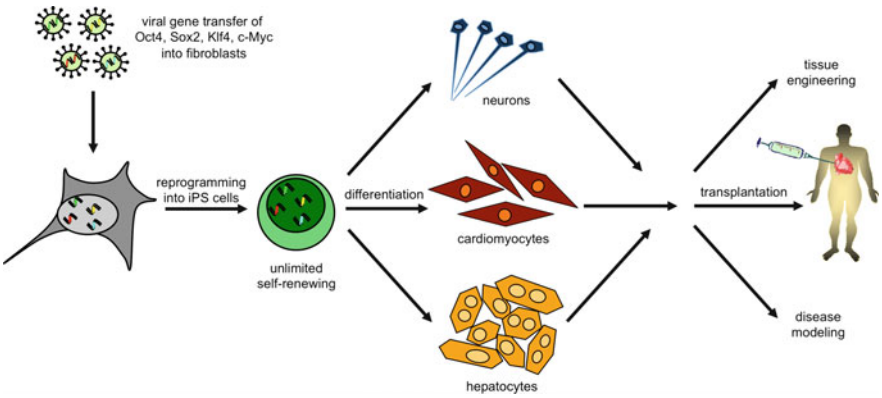

Fig. 1 Generation of patient-specific iPS cells. Patients' fibroblasts were cultivated and transduced with lenti- or retroviral vectors encoding the four reprogramming factors Oct4, Sox2, Klf4, and c-Myc. With a limited efficiency, few fibroblasts change their cellular fate and acquire an induced pluripotent stem cell phenotype. Applying *in vitro* differentiation protocols, cell derivatives of all three germ layers (like neurons, cardiomyocytes, and hepatocytes) can be generated. Those cells might resemble the patient's diseased cell phenotype and allow studies on new drug targets or pathophysiological mechanisms and may be used for tissue engineering or cell transplantation approaches

The generation of iPS with doxycycline (dox)-inducible reprogramming vectors from murine embryonic fibroblasts (MEFs) carrying an Oct4-GFP reporter gene knock-in allele enabled first insights into this process [40]. An exposure of dox for at least 8 days was necessary to obtain iPS cell colonies as analyzed after 20 days. The number of iPS cell colonies was higher after admission of dox for 10, 11, 12, and 13 days, respectively. Interestingly, during 12 days of dox-induction, the expression of the fibroblast marker Thy1 was decreasing, while the murine pluripotency-associated marker SSEA1 was increasing. The expression of SSEA1 was detectable earlier than the expression of the canonical pluripotency factors like Oct4 and Sox2 [40]. In a more advanced system, so-called dox-inducible secondary iPS cells were investigated by generating chimeric mice from dox-inducible iPS which carry a puromycin resistance [41]. MEFs from these chimeric mice were selected with puromycin, resulting in a MEF population which originated from one primary iPS cell line. Adding dox to these MEFs resulted in the generation of secondary iPS with a much higher efficiency, namely 4% compared to ~0.1% in primary iPS cells. However, the efficiency was far from reaching 100% as one could assume from the experimental outline. Two reasons for this discrepancy can be discussed as outlined by a recent comment of Yamanaka [42]. First, an elite cell population (such as a rare stem/progenitor cell) is more susceptible to iPS reprogramming and, therefore only a small subset of cells can be successfully reprogrammed into a pluripotent state. Second, stochastic genetic and epigenetic changes are mandatory for successful reprogramming, and this might only happen in a subset of cells. Based on recent literature, Yamanaka favored the latter explanation, which is further strengthened by

an elegant recent study: Hanna et al. utilized the dox-induction system for secondary iPS cells, but started with clonal pre-B-cells as a more homogenous starting population than MEFs [43]. Initially, only 3–5% of the cells gave rise to iPS colonies within 2 weeks. But eventually almost all of the cells committed to iPS colonies with latency times up to 18 weeks. These differences in latency were not predictable by any experimental parameter which is highly consistent with the necessity of yet unknown stochastic events during iPS cell reprogramming.

4 Technologies for Generation of Induced Pluripotent Stem Cells

The recent reprogramming of somatic cells into pluripotent ES-like cells [6, 7] is generally considered as a revolutionary breakthrough for the development of novel regenerative therapies. However, the initial technique was very inefficient and restricted to embryonic and adult fibroblasts as cell source. With respect to generation of clinically applicable cells, the classical technology based on retroviral overexpression of several reprogramming factors poses risks including the potential for insertional mutagenesis [44] and malignant transformation resulting from activation of oncogenic transgenes.

First reports on the induction of murine and human iPS cells reported reprogramming efficiencies of about 0.01–0.1%, resulting in relatively few fully reprogrammed cell clones. In the meantime, major improvements in reprogramming efficiencies have been achieved.

Recent results demonstrated reprogramming of a variety of cell types (for example, [45, 46]) including clinically easily accessible cell types such as keratinocytes, hair cells [47], and blood cells [48–51]. These results suggest that the majority of somatic cell types if not all cells can be reprogrammed. Efficiency of reprogramming could be dramatically increased up to ~2% for human cells [52] and up to 28% for secondary mouse iPS cells in an inducible transgenic mouse model [48]. In addition, it has been shown that depending on the cell type, and although very inefficient, iPS cells can be generated using only two [46] and even one reprogramming factor [53, 54].

These remarkable improvements have been achieved mainly through optimized reprogramming protocols, the use of siRNAs/shRNAs against p53/p21/UTF-1/DNA methyltransferase [55–57] and application of different small molecules for inhibition or activation of different factors and pathways (for review, [58] is recommended). These include inhibitors of histone deacetylase [59], the G9a histone methyltransferase [60], the TGFß- and MEK-ERK pathways [52], and an agonist of L-type calcium channels [61]. Inhibitors of GSK-3 [62, 63], MAP Kinase [63], and TGF-ß [64, 65] have been used to replace KLF4 [66] or SOX-2 (and c-Myc) [60, 63, 65]. Micro RNA (miR)-based approaches may represent another way to replace integrating vectors and recent publications indicated the usefulness of miR-302 and of the miR-290 cluster, the latter being downstream effectors of c-Myc, for reprogramming of somatic cells [67, 68].

Since most of the typically applied reprogramming factors including OCT4, SOX2, KLF4, MYC, NANOG, and LIN28 can be considered as oncogenes and may lead to malignant transformation of iPS-derivatives, permanent presence of those transgenes in the reprogrammed cells should be avoided and the development of transgene-free iPS cells is mandatory. In addition, insertional mutagenesis associated with integrating vectors may result in malignant transformation and loss of function. Thus, alternative approaches are desired for production of clinically applicable iPS cells, and very recent studies have already demonstrated the possibility of using conventional plasmids [69], nonintegrating adenoviral [70] and episomal vectors [71], as well as protein transduction [72, 73], instead of integrating vectors. Very recently, another paper demonstrated the generation of transgene-free human iPS cells by means of a vector system based on Sendai virus, an RNA virus without DNA state [74].

Although alternative approaches to induce pluripotent stem cells that avoid integration of transgenes into the host genome have now been demonstrated generally feasible, those methods are currently largely far from being technically mature: episomal approaches are extremely inefficient, genomic integration is not excluded, and oncogenes such as MYC and large T-antigen are required [71]. Protein transduction is also yet extremely inefficient and, importantly, requires huge amounts of recombinant proteins [72, 75].

Clearly, the above techniques are extremely promising; nevertheless, further significant improvement and development of novel and modified techniques is required.

5 Induced Pluripotent Stem Cells: Risks and Limitations

Although it is now generally accepted that iPS cells are pluripotent, it has been observed that individual iPS clones show considerable variation in their potential for differentiation. Whereas such variations can also be observed between different ES cell lines, variations between individual iPS cells clones may be even higher, especially due to incomplete transgene silencing, which apparently leads to delayed and less efficient differentiation [76]. Thomson et al. very recently reported a lower neural differentiation efficiency of a series of human iPS cell clones compared to several established ES cell lines [77]. Interestingly, this was also observed for transgene-free iPS cell clones generated by means of episomal vectors, thereby arguing for further reasons underlying the observed variations in differentiation behavior. Clearly, further work is needed to clarify whether iPS cells hold similar differentiation potential to ES cells and how the best iPS cell clones for a certain purpose can be identified.

Of major importance for future clinical application of iPS cells is to overcome current limitations such as lack of large scale culture technologies and inefficient specific differentiation, and to assess iPS cell related risks. As in case of ES cells, there are issues of teratoma formation after transplantation of iPS-cell derivatives

and of chromosomal abnormalities that could arise during stem cell expansion [78–82]. Whereas teratoma formation is considered to be manageable through suitable rigorous cell purification approaches and genetic systems enabling the ablation of contaminating cell grafts [83, 84], it is currently unknown whether and how chromosomal abnormalities which may result in malignant transformation can be avoided during extended cell expansion.

Another critical point is the use of oncogenic transgenes, such as MYC, for reprogramming and the risk of insertional mutagenesis due to the use of retroviruses to induce pluripotency. As discussed above, alternative technologies for generation of transgene-free iPS cells are thus crucial for clinical application of iPS-based cell and tissue transplants.

Another aspect regarding the production of clinically useful iPS cells concerns the quality of iPS cells derived from somatic cells of aged individuals. Although mammalian species differ dramatically with respect to their maximum life span and the incidence of spontaneously occurring tumors, a common observation from mouse to man is that the risk of cancer increases exponentially during the later stages of life [85, 86]. In general, epigenetic [87] and genetic modifications, including telomere shortening and spontaneous mutations, are considered as underlying causes. For example, somatic mutations of the epidermal growth factor receptor have been shown to lead to the development of non-small-cell lung cancer [88].

On the other hand, mitochondrial mutations typically have effects on catabolism and cell function. Normal mitochondria help to remove free radicals, but somatic mutations of the mitochondrial DNA over time make them less effective and, thus, may contribute to the advancement of aging and/or cancer [89].

Whereas epigenetic changes and loss of telomerase activity in cells of aging individuals may be reversed during induction of pluripotent stem cells [90], acquired chromosomal abnormalities and/or point mutations are not corrected during reprogramming and may lead to iPS-derivatives with reduced functionality. In addition, somatic cell clones with acquired mutations that result in higher reprogramming efficiency and increased proliferation rates are likely to become enriched during expansion of the primary cell source. This is further enhanced during the reprogramming and proliferation of the resultant iPS cells, thereby supporting an increased cancer risk.

As a consequence, one should consider the use of "young" cell sources such as cord blood [49] for derivation of clinically useful iPS cells.

6 Induced Pluripotent Stem Cells for Drug Screening and Safety Pharmacology

Pluripotent stem cells, with theoretically unlimited potential for proliferation and differentiation, may not only represent a cell source for basic biomedical research and clinical cell transplantation, but are rather considered as the most important

prerequisite for the development of novel, high-throughput assays for drug screening and pharmacology studies.

During the first phase of drug development, the most potent compounds are identified among several hundred thousands of candidates, followed by the detailed characterization of selected compounds (several hundred to several thousand) in primary and secondary pharmacology studies. Finally, safety pharmacology studies focus on identifying adverse effects on physiological functions.

The cost-effective and available high-throughput assays used in the early phases of drug screening do not always meet the data quality requirements for detailed characterization of pharmacodynamic properties and potential of undesired side effects. Indeed, data of higher quality can be generated only through the use of sophisticated, costly, and labor-intensive in vitro assays or by *in vivo* experiments, for example telemetry studies. Due to high costs of animal experiments, safety pharmacology studies, required by law, are usually completed in the final phases of drug development. As non-mammalians and rodents poorly reflect specific aspects of human physiology and immunology, large animals such as dogs and NHPs are commonly used in the last phase of preclinical pharmacology studies and safety pharmacology.

In contrast, over the past few years, safety pharmacology studies have been initiated earlier in drug discovery as a way to reduce the rate of failure and thereby costs. However, to further support this process, as well as to reduce the number of ethically problematic animal experiments, it is now required to develop cost-effective *in vitro* assays producing higher quality data than currently available.

Current assays for cardiac safety pharmacology represent a common example for pharmacological screening systems. Available assays for cardiac safety pharmacology can be separated into three classes: (1) relatively cost-effective assays more or less suitable for automated high-throughput screening, but with limited predictive value, for example, the dofetilide binding assay or rubidium efflux assay; (2) labor (and cost) intensive *in vitro* assays with higher predictivity, such as Langendorf heart and patch clamp; and (3) most expensive, animal experiments in dogs or monkeys, but with the highest predictive value.

One major problem of all cardiac *in vitro* assays is the cell source. Human CMs would be optimal; however, these are not available as myocyte-derived tumor cell lines and adult primary CMs lose proliferation potential. As an alternative, existing assays use *Xenopus* oocytes or human tumor cell lines genetically modified to express hERG channels [91], or primary CMs prepared from hearts of other species, for example dogs. However, the phenotype of these cell sources is far from being able to mirror closely the function of human CMs.

The availability of ES cells and iPS cells from humans with their high expansion capacities now offers the possibility to generate almost unlimited numbers of functional CMs [49] as the perfect tool for the development of novel high-throughput pharmacological screening systems. Such assays can be based not only on electrophysiological detection of prolongation of QT-intervals, but also on detection of Ca^{2+}-transients or the biochemical/biophysical analysis of specific ion channels. Furthermore, the influence of drugs on cardiovascular differentiation and

development [92] can be tested by means of pluripotent stem cell lines, transgenic for fluorescent reporter genes under control of specific promoters.

In case of screening for prolongation of the QT-interval [93], pluripotent stem cell-derived CMs are probably better qualified than adult CMs, from a functional point of view, as they represent embryonic CMs with a typical reduced repolarization reserve similar to CMs of diseased hearts. Therefore, prolongation effects on repolarization can be detected at much lower concentrations as compared with cells from healthy adult tissue. This may even be an advantage over animal experiments and first stages of clinical studies where as, in these cases, usually healthy individuals are tested.

In addition to iPS-derived CMs from healthy individuals, iPS-derived CMs from patients with genetically based diseases, for instance from long QT patients, may be highly useful for drug screening purposes. Although not shown so far for iPS-derived CMs from long QT patients, it is supposed that such cells are more sensitive to certain QT-interval prolonging drugs than control cells.

Similar to cardiac drug screening, the cell source represents one if not the bottleneck for development of novel *in vitro* assays in other fields, for instance, safety screening for hepatotoxicity. So far, only limited numbers of human hepatocytes have been available from donor organs that are unsuitable for clinical organ transplantation, and unlimited supply with iPS cell-derived functional hepatic cells would overcome the major bottleneck of in vitro drug evaluation of hepatotoxicity. Recently, two groups described successful adaption of human ES cell differentiation protocols to human iPS cell lines, which give rise to hepatic cells exhibiting all major metabolic liver functions [94, 95].

7 Induced Pluripotent Stem Cells for Disease Modeling

The generation of human iPS cells from various types of somatic cells provides improved iPS cell generation strategies for adequate patient-specific cell culture models for a variety of diseases and disorders, including hematopoietic disorders, neurological disorders, arrhythmic heart disorders, pulmonary diseases, and metabolic liver diseases (Fig. 1). Implications of the genetic defect during the specification of the affected cell type can be investigated and the severity of the defect can be correlated to the individual course of the disease. Most importantly, derivatives of disease-specific iPS offer an unlimited cell resource for *in vitro* studies allowing not only advanced studies on the pathophysiology of such diseases but also evaluation of future therapeutic interventions, including gene therapeutic approaches.

With respect to patients suffering from myeloproliferative disorders (MPDs), studying disease-specific iPS cells might be of particular interest if the disease was caused by a specific genetic mutation. Ye and colleagues recently described the generation of iPS cells from patients' CD34-positive blood cells that carry the JAK2-V617F mutation leading to MPD [96]. These MDP-iPS were morphological undistinguishable from normal human iPS cells and did not show alterations with

respect to their pluripotent phenotype. Nevertheless, *in vitro* differentiation into blood cells demonstrated an increased erythropoiesis, resembling the primary disease of the patients [96]. In a recent letter, a Chinese group reports on the generation of iPS cells from patients suffering from β-thalassemia, which is an inherited disease characterized by reduced synthesis of hemoglobin beta subunit [97], but the authors did not provide analyses of the diseased phenotype after *in vitro* erythropoiesis.

Aiming at neurological disorders, numerous groups are interested in studying iPS from patients, who suffer from an inherited form of amytrophic lateral sclerosis (ALS). One future goal might be to generate patient-derived transplantable motor neurons but today's efforts focus on modeling the disease phenotype by analyzing and influencing the motor neuron destruction. The first crucial step for those studies has already been achieved by generating ALS-specific iPS cell lines that were differentiated into motor neurons in vitro [98].

Spinal muscular atrophy is a genetic disease affecting motor neurons, which, in contrast to ALS, leads to symptoms in early childhood. In an elegant study, Ebert et al. described the generation of disease-specific iPS cells from patients' skin fibroblast and compared these cells with iPS cells derived from fibroblasts of the unaffected mothers [99]. Importantly, the authors were able to demonstrate that the patient iPS cell-derived motor neurons showed selective deficits and, thereby, maintained the disease phenotype.

One pitfall of using iPS cells for disease modeling might be that the cells acquire mutations in relevant pathways due to insertional mutagenesis caused by the retroviral delivery of the reprogramming factors. This issue is addressed in one study on iPS cells that were derived from five individual patients suffering from Parkinsons disease [100]. Using Cre-excisable reprogramming factors, the authors generated factor-free iPS cell lines that were a superior source of cells for studying iPS cell-derived dopaminergic neurons. A very rare disease of the peripheral nervous system was studied using iPS cells derived from patients' fibroblasts suffering from familial dysautonomia, FD [101]. A point mutation in the IKBKAP gene results in mis-splicing, but to date little is known about the detailed mechanism of the loss of autonomic and sensory neurons in the peripheral nervous system. FD-derived iPS cells could be differentiated into peripheral neurons, which mimic the underlying disease phenotype by showing alterations in the levels of normal IKBKAP transcripts and marked defects in neurogenic differentiation and migration behavior. Moreover, FD-iPS cells were used to evaluate candidate drugs such as kinetin, epigallocatechin gallate, and tocotrienol.

Besides hematologic and neurologic disorders, iPS cells were also generated to study metabolic diseases, such as type 1 diabetes mellitus. In recent years intense basic science has led to improved protocols to differentiate human ES cells into insulin-producing β-cells [102, 103] but still more insights with respect to the (auto-) immunologic reactions causing the loss of β-cells are desired. The lack of available patient-derived type 1 diabetes (T1D)-specific β-cells is regarded as one of the major obstacles that limit the current knowledge of the disease mechanism. Maehr and colleagues from Doug Melton's lab were demonstrating that T1D-iPS could be generated for various patients and could be differentiated into insulin-producing

cells [104] and, thus, might be the long sought source not only for T1D disease modeling but also for future cell replacement therapies.

8 Induced Pluripotent Stem Cells for Cell-Based Therapies

The above-mentioned iPS cells from T1D provide a good example for the two aspects of iPS cell research in regenerative medicine. Besides indirect use of iPS-cell derivatives to study pathophysiology and new pharmacotherapeutic strategies of the respective disorder, patient-specific iPS cells would be a unique resource as therapeutic cell transplants if mature and functional cell derivatives were obtainable by in vitro differentiation. According to the efforts of various laboratories as well as of the company Novocell Inc., it might well be that β-cells from diabetic patients' iPS cells are the first autologous iPS-derivatives to be used in clinical applications. Hereby, one major concern reflects autoimmunologic depletion of the patients' iPS cell-derived cell transplant, which might be overcome by encapsulation of the transplanted cells into alginate-based matrices [105].

Again, the first proof-of-principle for iPS cell-based therapies was given in the field of hematology using a humanized sickle cell anemia mouse model. After generation of iPS cells from these mice, the autologous iPS cells could be genetically repaired by correction of the human sickle hemoglobin allele applying gene-specific targeting. Moreover, by *in vitro* differentiation, disease-free hematopoietic progenitor cells could be obtained, which were able to ameliorate the phenotype after transplantation into the diseased mice [106].

Even if therapeutic applications with human iPS cells need to overcome various technical and safety issues, a lot of other diseases could be candidates for iPS-derived cell therapies. However, besides safety issues while generating iPS cells, one might also take into account that some genetic disorders will require genetic correction of the primary donor cells prior to iPS reprogramming. This was the case when Raya and colleagues from Belmonte's lab [107] were attempting to generate iPS cells from patients suffering from Fanconi anemia (FA). Due to the chromosomal instability of the primary FA-fibroblasts the authors were unsuccessful in generating FA-specific iPS cells. Only cells that were corrected using a normal copy of the *FANCA* gene gave rise to iPS cell lines that could be expanded and pass all criteria for human pluripotent stem cells. Furthermore, these cells did not show major abnormalities during *in vitro* hematopoiesis, suggesting their future use in therapeutic applications once the above-mentioned safety issued are sufficiently resolved.

As mentioned above, human iPS cell-derived hepatic cells can be obtained if suitable differentiation protocols are applied [94, 95]. Besides their use for pharmacologic applications such as drug screening and toxicological analyses, hepatic iPS-cell derivatives might become a valuable autologous source for cell therapies of acute liver failure or of metabolic liver diseases. However, refined differentiation protocols need to be established that result in a more mature hepatic phenotype,

which can efficiently engraft and repopulate diseased livers. As pointed out in a recent study taking advantage of a murine competitive liver repopulation assay, human ES cell-derived hepatic cells failed to give rise to a detectable amount of hepatic cells in this xeno-transplantation model [108].

Innovative concepts in treating retinal degeneration focus on stem cell-derived photoreceptor progenitor cells or retinal pigmented epithelial cells. In a recent study, Osakada et al. describe the generation of retinal progenitor cells from both human ES cells and human iPS cells, using a small molecule-based differentiation protocol that avoids cross-species contaminations as observed if bacterial or animal products were used [109]. Such xeno-free iPS cell-derived retinal progenitors may be highly useful for clinical translation of therapeutic concepts as developed by Lamba and colleagues, who transplanted human iPS-derived retinal epithelial cells into mice and were able to demonstrate engraftment of human cells in the mouse retina and show expression of photoreceptor markers [110].

Whether finally patient-specific iPS cells or allogeneic iPS will be clinically applied is not clear at present. Certainly, autologous cells are advantageous since pharmacological immunosuppression is not required. However, if patient-specific iPS-derivatives are applied, the required time frame for isolation and culture of primary cells, reprogramming, selection of suitable clones, expansion, differentiation, enrichment, and optionally tissue engineering has to be considered and will exclude treatment of acute diseases and injuries. Maybe worldwide banking of allogeneic iPS(-derivatives) may provide the required cells at least for the majority of patients. Other possibilities are the engineering of genetically modified "universal" cell lines or the development of clinically applicable iPS-based tolerance induction protocols. In both cases, cell production would be possible on an industrial scale, thereby leading to dramatically reduced costs compared to autologous cell therapy.

9 Perspectives

The generation of human iPS cells [6, 8, 111], together with the latest developments showing production of iPS cells without integrating vectors [69–71, 75], create new opportunities for the establishment of clinically useful autologous stem cell lines (Fig. 1).

After these pioneering developments, four major issues need to be addressed. First, it is now crucial to develop technologies that enable selection of the "best" ones from the large number of iPS clones that usually result from one reprogramming experiment: besides culture characteristics, the potential for teratoma formation and potential predetermination to differentiate in the desired lineage(s) are critical for clinical application. Second, it is mandatory to improve the efficiency and specificity of *in vitro* differentiation in order to obtain iPS-cell derivatives that are mature enough to mimic the targeted (diseased) cell type during drug screening and toxicology analyses. Third, besides differentiation strategies, functional engraftment capabilities

of transplanted human iPS-cell derivatives need to be addressed. Finally, and probably most critical, several safety issues need to be resolved, because the iPS cells themselves and their differentiated derivatives might harbor various genetic and epigenetic abnormalities, which could be acquired just during the reprogramming process or selected during expansion of the (most proliferative) cell clones.

In conclusion, iPS cell biology is a young field within stem cell research that covers various important and attractive scientific areas, ranging from basic understanding of (epi-)genetics during nuclear reprogramming, over applied sciences with respect to stem cell expansion and differentiation, to translational research on clinical applications in (large) animal models in preparation for future phase-I clinical trials.

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Induced Pluripotent Stem Cell Technology in Regenerative Medicine and Biology

Duanqing Pei, Jianyong Xu, Qiang Zhuang, Hung-Fat Tse, and Miguel A. Esteban

Abstract The potential of human embryonic stem cells (ESCs) for regenerative medicine is unquestionable, but practical and ethical considerations have hampered clinical application and research. In an attempt to overcome these issues, the conversion of somatic cells into pluripotent stem cells similar to ESCs, commonly termed nuclear reprogramming, has been a top objective of contemporary biology. More than 40 years ago, King, Briggs, and Gurdon pioneered somatic cell nuclear reprogramming in frogs, and in 1981 Evans successfully isolated mouse ESCs. In 1997 Wilmut and collaborators produced the first cloned mammal using nuclear transfer, and then Thomson obtained human ESCs from in vitro fertilized blastocysts in 1998. Over the last 2 decades we have also seen remarkable findings regarding how ESC behavior is controlled, the importance of which should not be underestimated. This knowledge allowed the laboratory of Shinya Yamanaka to overcome brilliantly conceptual and technical barriers in 2006 and generate induced pluripotent stem cells (iPSCs) from mouse fibroblasts by overexpressing defined combinations of ESC-enriched transcription factors. Here, we discuss some important implications of human iPSCs for biology and medicine and also point to possible future directions.

Keywords Disease modeling, Embryonic stem cells, Induced pluripotent stem cells, Regenerative medicine, Reprogramming

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Abbreviations

bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
EpiSCs	Epiblast stem cells
ERK	Extracellular signal-regulated kinase-1
ESCs	Embryonic stem cells
GFP	Green fluorescent protein
GSK3B	Glycogen synthase kinase 3 beta
ICM	Inner cell mass
iPSCs	Induced pluripotent stem cells
LIF	Leukemia inhibitory factor
Oct4	Octamer-4
POU5F1	POU class 5 homeobox 1
SCNT	Somatic cell nuclear transfer
SKOM	Sox2, Klf4, Oct4, c-Myc
SKONL	Sox2, Klf4, Oct4, Nanog, Lin28
STAT	Signal transducer and activator of transcription
Tgfb	Transforming growth factor beta

1 Pluripotency and Induced Pluripotency

1.1 Embryonic Stem Cells

After fertilization the mammalian zygote undergoes a series of quick symmetric cell divisions to reach the morula stage. Soon afterwards the first differentiation event produces the blastocyst, which is composed of an outer layer or trophectoderm and the inner cell mass (ICM) [1] (Fig. 1). The blastocyst stage embryo implants into the receptive uterine wall and then the trophectoderm transforms into the placenta, which connects the developing fetus to the maternal uterine wall and is responsible for the exchange of nutrients and oxygen. The ICM transform into the

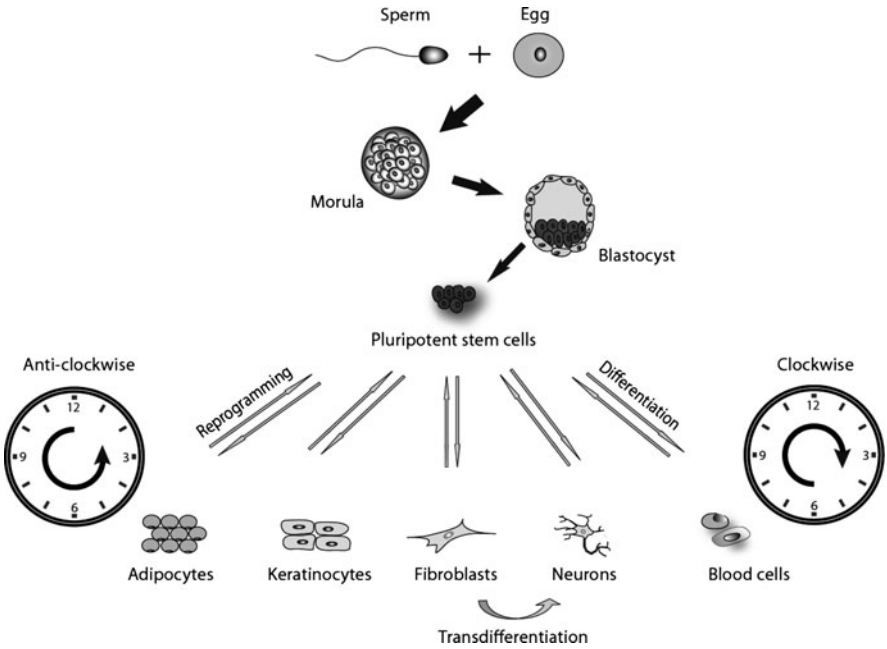

Fig. 1 Schematic representation of embryonic stem cell differentiation and reprogramming

epiblast, which later differentiates into the three germ layers (ectoderm, mesoderm, and endoderm) through a process known as gastrulation, and these three lineages form all the tissues of the newborn individual [2]. Embryonic stem cell (ESC) lines are considered in vitro representations of the ICM, and they are derived from preimplantation blastocysts once these are broken and the cells cultured in specific tissue culture conditions [3, 4]. Human ESCs are phenotypically and functionally very distinct from mouse ESCs; for example, they are flat and require basic fibroblast growth factor (bFGF) and Activin A/transforming growth factor beta (Tgfb) signaling to maintain their pluripotent state, whereas mouse ESCs are tightly clustered and require leukemia inhibiting factor (LIF)/Stat3 and Bmp4 signaling [5–7]. Human ESCs also differ epigenetically from mouse ESCs by several criteria such as X chromosome inactivation, their pattern of gene expression, and pluripotency factor promoter occupancy across the genome [8]. A different type of stem cells termed epiblast stem cells or EpiSCs can also be derived from the postimplantation mouse epiblast, and these cells share many characteristics with human ESCs including the flat morphology and tissue culture requirements [9, 10]. EpiSCs have a very restricted developmental potential but they can produce teratomas composed of the three germ layers. Interestingly, they can be reversed into bona fide ESCs by manipulating the culture conditions or using chemical inhibitors [8, 11]. Altogether these findings have provoked questions concerning the true identity of human ESCs and whether they can also be reset to a mouse ESC-like status. In mouse,

pluripotency is routinely tested by injecting cells into blastocysts of a mouse strain with different coat color, but this approach cannot be used in humans and thus many questions remain. The failure to isolate true ESCs from other relevant mammalian species like ungulates (e.g., pig) [12] and even from many (defined as non-permissive) mouse strains [8] has also been a major roadblock in making cross-comparisons. Nevertheless, given their ability to differentiate into all possible lineages of the body, human ESCs have long been viewed as a potential "fountain of youth" for regenerative medicine purposes and a major scientific advance [13] (Fig. 1). Adult stem cells of mesenchymal origin have also raised much interest for transplantation purposes and are poorly immunogenic, but they have a rather limited differentiation potential and are difficult to expand ex vivo [14].

1.2 Pluripotency and Its Regulators

Despite the immense potential of human ESCs, the use of human embryos, even if from in vitro fertilization, remains controversial, and the problem of immune rejection following transplantation in patients is difficult to solve [15]. This has stimulated scientists to find alternative ways to produce pluripotent cells in vitro by resetting the nuclei of somatic cells to an embryonic-like stage. These methods are generically termed nuclear reprogramming or reprogramming, and the two most extended variants are somatic cell nuclear transfer (SCNT) [16] and direct reprogramming using exogenous factors [17, 18]. Pioneer studies by King, Briggs, and Gurdon had shown decades earlier that when an undifferentiated [19] or a somatic nucleus [20] is transferred to a frog egg deprived of its own nucleus, the egg bearing the exogenous DNA can produce a normal tadpole. This was proof of concept that developmental fates are not a fixed state and suggested that somatic cells contain all the necessary information to direct the development of a new individual. The successful cloning of Dolly in 1997 proved this idea and made human SCNT a top scientific objective for producing patient specific human ESC-like pluripotent cell lines. But even though SCNT was successful in a number of other species including non-human primates [21, 22], many technical challenges persist in humans and early reports turned out to be fraudulent. After this, thanks at least in part to improved technologies for high throughput functional screening, studies worldwide progressively narrowed into the identification of key transcriptional networks that govern ESC function [23, 24]. Given the existing restrictions in many countries, most of these analyses were done with mouse ESCs, but the existing paradigms apply to a great extent in humans and possibly other mammals as well. Among other key transcription factors, Octamer-4 (Oct4), identified by Austin Smith and collaborators [25], and Nanog, identified by Austin Smith [26] and Shinya Yamanaka [27], are essential regulators of pluripotency. For example, levels of the homeobox-containing protein Oct4 (also termed POU5F1) only 50% higher than normal induce mesodermal differentiation, while if 50% lower ESC fate is shifted towards the trophectoderm. Likewise, knock-down of Oct4 prevents

proliferation of ICM cells and induces differentiation into trophectoderm in mouse embryos [25]. In contrast, the homeodomain containing protein Nanog is not absolutely required to sustain mouse ESC characteristics [28], but its overexpression renders them resistant to differentiation upon LIF withdrawal [26–28]. Oct4, Nanog, and other ESC transcription factors coordinately bind to DNA-binding sites in target promoters along the genome [24], and act as gene activators or repressors depending on the identity of extra proteins that are recruited to these promoters; for example, recruitment of the transactivator P300 associates with active transcription, and proteins of the Polycomb group with repression [29, 30] (Fig. 2). This duality has the purpose of coordinating the activation of pluripotency genes with the silencing of others that are involved in lineage differentiation programs, and is tightly related to the nature of the concomitant histone modifications (e.g., acetylation, methylation) [31]. In addition, ESC transcription factors usually bind to their own promoters in an autoregulatory loop, and can induce the transcription of each other [24]. The accessible amount of information regarding ESC pluripotency is nowadays impressive, and although knowledge is not yet fully digested, it was determinant to allow Takahashi and Yamanaka to generate induced pluripotent stem cells (iPSCs) from mouse somatic cells in 2006 [17], an outstanding achievement.

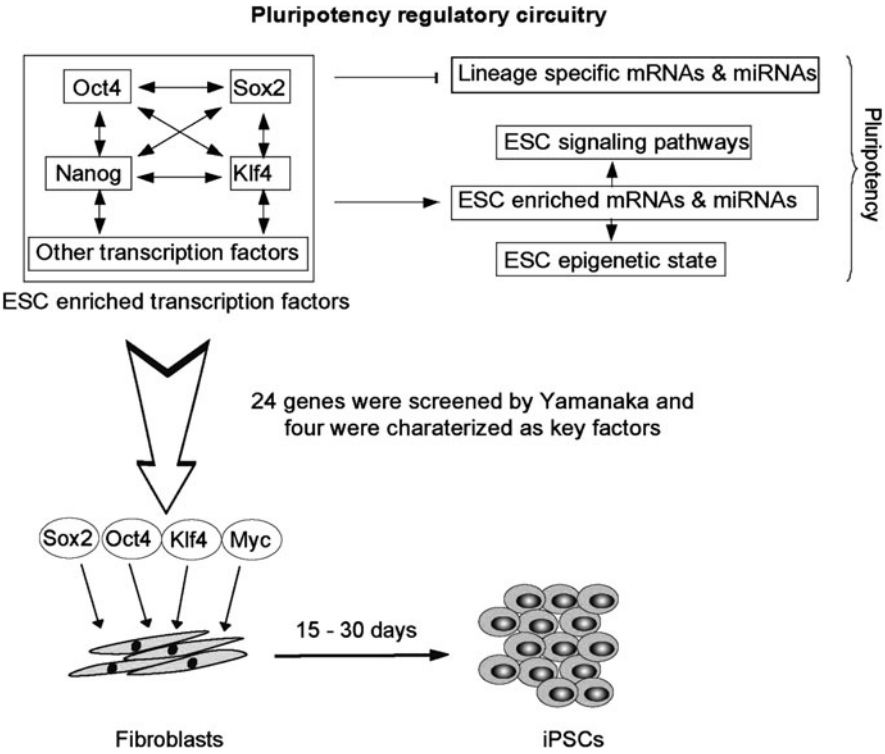

Fig. 2 Schematic representation of the transcriptional networks controlling ESC pluripotency and how this knowledge was employed to discover iPSCs

1.3 Induced Pluripotent Stem Cells

Takahashi and Yamanaka had a simple yet sophisticated approach to nuclear reprogramming: they first selected a combination of transcription factors and other proteins with well established roles in ESC behavior, which were delivered as a pool into mouse fibroblasts by means of retroviral vectors (Fig. 2). The transduced cells were cultured in conditions similar to mouse ESCs and after approximately 15 days colonies with mouse ESC characteristics formed; then the exogenous factors were eliminated one by one until it appeared that the SKOM (Sox2, Klf4, Oct4, and c-Myc) cocktail was necessary and sufficient (Fig. 2) [17]. The first generation iPSCs formed teratomas but had a different global gene expression pattern from ESCs and failed to produce adult chimeric mice. The use of genetically engineered knock in mice with reporter systems (green fluorescent protein, GFP) and resistance to antibiotics inserted into the promoter of key ESC pluripotent regulators (e.g., Nanog) allowed the generation of chimera competent iPSCs with germ line transmission by the Yamanaka and Jaenisch labs [32, 33], and ever since the field has been an explosion of remarkable achievements one after the other. In particular, the Yamanaka and Thomson laboratories were the first to produce human iPSCs using retroviral or vectors and SKOM [34] or SKONL (NL stands for Nanog and Lin28) factor combinations [35].

1.3.1 Delivery Methods

Methods for generating iPSCs are evolving very quickly and the choice is very varied but can basically be divided into integrating and non-integrating approaches. The initial experiments by Takahashi and Yamanaka used retroviral vectors [17], and this turned out to be particularly useful given that ESCs have self defense mechanisms (DNA methylation of the integrated virus) against invading genomes such as retroviruses. Accordingly, silencing of the exogenous retroviral vectors was established as a relevant criterion to discern fully reprogrammed from partially reprogrammed colonies [17]. iPSCs have also been generated with lentiviruses, which have a less reliable degree of silencing in ESCs/iPSCs but can be combined with an inducible doxycycline-dependent system. Retroviral and lentiviral approaches, although robust and reproducible, have the problem of possible reactivation of the viral vector, in particular after transplantation, and for example mice generated with SKOM retroviruses had a high frequency of tumors and other abnormalities [32]. This is possibly related to c-Myc and over time the need for this oncogene (and other factors as well) in the cocktail has been bypassed [36, 37], but we should not forget that in some instances Klf4 has also been regarded as an oncogene and overexpression of Oct4 in adult tissues can cause dysplasia [38, 39]. To avoid this problem, Jaenisch and collaborators induced iPSCs using a polycystronic cassette that could be removed by adding CRE recombinase [40]. Interestingly, the authors found a change in gene expression in iPSC cell lines before and

after excision with the recombinase, which points to minor presence of the viral transcripts having a substantial impact on gene expression. Nevertheless, this approach, although appealing, leaves a genetic scar after the excision and still does not preclude the risk of insertional mutation. Hochedlindger and collaborators also made mouse iPSCs using adenoviruses [41] and afterwards this was achieved in human cells [42]. More recently human iPSCs were produced by Yu et al. using episomal vectors [43], and mouse and human iPSCs by Zhou et al. and Kim et al. using proteins [44, 45]. These non-integrating approaches have very low efficiency compared to retroviruses/lentiviruses and the challenge is to improve the reproducibility of existing protocols. The addition of compounds such as the histone deacetylase inhibitor valproic acid [46], vitamin C [47], or chemical inhibitors of Tgfb receptors [48–50], and a careful donor cell selection will definitely facilitate this objective. The number of cell types that can be used to generate iPSCs is growing steadily [51–56]. So far, superior cell sources are defined mainly on the basis of such a weak criterion as human ESC-like morphology and alkaline phosphatase staining, but this may be misleading and it is important to evaluate the epigenetic reprogramming and safety of the resulting colonies using more accurate methods (see Sect. 1.3.3 below). Understanding why some cells are more amenable to reprogramming than others and how these compounds work will also shed light into the reprogramming.

1.3.2 Modeling Human Disease with iPSCs

Mouse transgenic and knock out models are extremely valuable for studying human disease but in many cases the parallelism between both species does not exist due to differences in animal physiology or in gene function. This has made it increasingly necessary to develop more accurate human disease models for mechanistic studies and drug discovery. One possible way to do this is using in vitro fertilized ovules after the corresponding preimplantation genetic diagnosis. This has produced human ESCs from diseases such as cystic fibrosis [57] or Huntington disease [58], but is severely constrained by ethical considerations and the diseases that are routinely screened. Another option is to modify genetically existing human ESC cell lines by means of homologous gene recombination, but apart from ethical concerns this area of research has been largely stalled due to technical difficulty in achieving DNA recombination compared to mouse ESCs [59, 60]. In this regard, if the targeting efficiency is low for knocking out one gene, it is almost negligible for eliminating the two. This approach may still be feasible for X chromosome-linked syndromes, in which only one allele needs to be abrogated, for example Lesch–Nyhan disease [61], but among other considerations the selection procedure may alter the epigenetic state and quality of the resulting ESC cell lines. More recently, successful homozygous gene disruption in human ESCs using zinc-finger nuclease-mediated genome editing [62] or a bacterial artificial chromosome (BAC)-based targeting approach has also been reported [63]. Regarding the zinc-finger nuclease

technology, not every gene is susceptible and the DNA-binding specificity of the designed zinc-finger proteins remains to be validated with vigorous genomic analysis. The generation of iPSCs from individuals with genetic diseases could solve these problems but caution is needed as there are also potential caveats [59, 60]. One fundamental consideration is that many diseases have a late onset and the neurons or other progeny derived from iPSCs may not reproduce the age related phenotype. In addition, some diseases are non-cell autonomous and require not only time to develop but also the existence of a body context (e.g., neurons affected by secretions of glia cells). Another perhaps more incapacitating problem is that differentiation protocols are still inefficient and the lack of a homogeneous population can be a major problem for detecting biomarkers and performing drug screening or transcriptomic/proteomics analysis. Nevertheless, recent reports have succeeded in finding either an in vitro phenotype or using patient-specific iPSC cell line generation to shed light onto the reprogramming. For example, Ebert et al. produced iPSCs from spinal muscular atrophia [64], Lee et al. from familial dysautonomia [65], and Agarwal et al. from dyskeratosis congenita [66], and this list is likely to increase steadily. Although setting up meaningful in vitro models will likely take several years and for many diseases it may never be achieved, this research will revitalize the interest on rare human conditions touching essential aspects of human physiology (e.g., DNA repair) for which the availability of patients (not to mention the tissue) is reduced.

1.3.3 Accuracy of the Epigenetic Reprogramming

iPSCs have been repeatedly described as identical or almost identical to ESCs but the initial comparisons were too vague for a matter of such importance and defining the epigenetic identity of iPSCs compared to ESCs is now a very active aspect of research. Among other major questions (Fig. 3) that are steadily discussed in all forums we have: is the epigenetic reprogramming of iPSCs complete or only an effective makeup? If it is only a makeup, then – as long as it works and is safe – does it matter? Also, is there any epigenetic memory from the tissue of origin and does this memory have any functional implications? Related to the latter, an interesting possibility is that the existence of a tissue-specific epigenetic memory confers an advantage rather than being negative, which could thus be exploited to develop iPSCs that retain a relevant functional ability. On the other hand, it could happen that the abnormal epigenetic reprogramming (either epigenetic memory or of a different kind) is a requisite for generating iPSCs. As was discussed above, in the mouse it is easy to test for the acquisition of pluripotency and recently adult animals were produced entirely from mouse iPSCs by means of tetraploid complementation [67–69]. This suggests that mouse ESCs and iPSCs are either epigenetically identical or that putative abnormally reprogrammed genes are not functionally relevant. However, this procedure still has a very poor success rate and it remains to be found whether these animals or their progeny are exempt of any physiological abnormalities. In this regard, for example, mice produced by SCNT are more prone

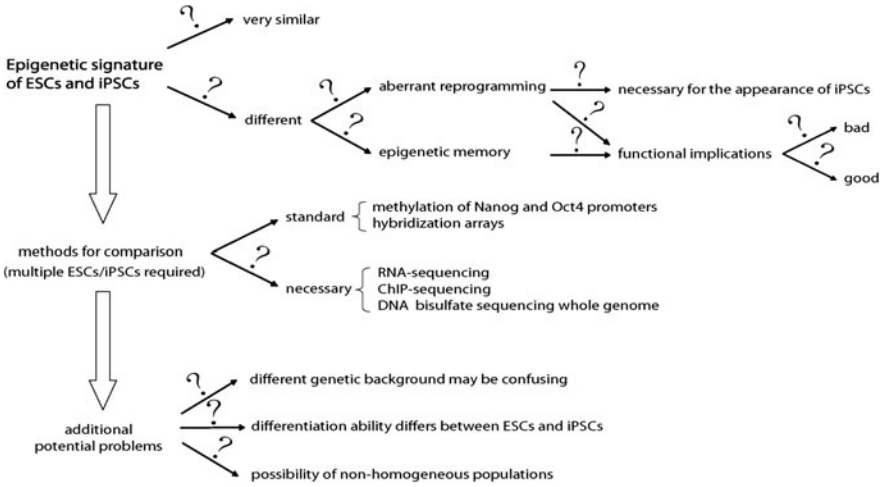

Fig. 3 Possibilities regarding the extent of the epigenetic reprogramming in iPSCs

to disease and can have developmental abnormalities [70, 71]. In the case of human iPSCs, epigenetic reprogramming is normally defined by complete DNA demethylation of selected regions of Oct4 and Nanog promoters and by hybridization arrays that compare the gene expression profile (DNA or microRNA microarrays) and the pattern of histone modifications (mainly histone methylation). Interestingly, a recent meta-analysis of published DNA microarrays by Chin et al. showed that mouse and human iPSCs retain a common gene expression signature especially during the first passages [72], and Ghosh et al. showed the retention in iPSCs from different tissues of patterns of gene expression reminiscent of the tissue of origin [73]. Both studies have compared iPSCs generated by different methods in different laboratories and although the conclusions are attractive their analysis is not exempt of problems. For example, Chin et al. defined abnormal reprogramming as those genes changed more than 1.5-fold between the average of a panel of iPSCs and a panel of ESCs [72], which can be misleading because gene expression of ESCs is known to differ between cell lines and so is expected of iPSCs. Besides, although they identified up-regulated genes that belong to developmental pathways, this could be a consequence of partial differentiation in the borders of some colonies, which for example is not infrequent during the first passages of freshly isolated human iPSCs. Ghosh et al. also stated that the retention of a footprint from the tissue of origin could be due to those iPSC cell lines being a heterogeneous population of both reprogrammed and partially reprogrammed cells [73]. In any case, DNA arrays have limitations, and a rather more accurate comparison should require digital sequencing technologies: deep transcriptomic sequencing, and whole genome ChIP-on-Chip sequencing and DNA bisulfate sequencing for assessing DNA methylation. The latter was recently achieved with human ESCs and these available data are a powerful resource for future comparisons with human iPSCs [74]. On the other hand, Doi et al. also used comprehensive high throughput

array-based relative methylation (CHARM) to identify in a more restricted part of the genome series of differentially methylated regions (DMRs) between multiple ESCs and human iPSCs and their respective donor cells [75]. Pick et al. also described the inadequate maintenance of imprinted genes, which was demonstrated by abnormal DNA methylation of their respective promoters, between donor cells and some of the resulting human iPSCs cell lines [76].

1.3.4 iPSCs from Other Species

After the existing technical hurdles and safety concerns are solved, the jump of human iPSCs to the first clinical trials will be a monumental step that cannot be made without prior animal validation. Given its ease and reproducibility, mouse iPSCs are unquestionably the preferred tool for mechanistic studies and technical innovations that subsequently become validated in the human model. Besides, proof of the principle that iPSCs have huge therapeutic potential was achieved early by the Jaenisch laboratory, which showed that mouse iPSCs from a mouse with sickle cell anemia can be used to correct the mutation using homologous differentiation followed by hematopoietic progenitor differentiation and transplantation [77]. However, in general the differences in size, physiology, and life span between mice and humans are too big for valuable comparisons. For example, the heart beat frequency in mice is several hundred per minute compared to around 70 in humans, challenging if not invaliding any possible conclusions made after iPSC derived-cardiomyocytes transplantation. This has encouraged researchers to develop iPSCs from other mammalian species, specifically the rat, monkey, and pig in this order. Rat iPSCs were generated by two independent groups [78, 79] following the successful isolation of rat ESCs using extracellular signal-regulated kinase-1 (ERK) and glycogen synthase kinase 3 beta (GSK3B) inhibitors by Smith and Ying [80, 81]. Li et al. used fibroblasts and SKOM retroviruses [78], while Liao et al. used fibroblasts and bone marrow mesenchymal cells infected with inducible lentiviruses [79]. In both studies, rat iPSC pluripotency was demonstrated by teratomas, and the formation of chimeric animals (without germ line transmission) was only reported by Li et al. [78]. Rats are larger than mice and, although their life span and physiology also differ from humans, they are excellent laboratory animals for a wide range of diseases. Monkey iPSCs were then produced from Rhesus monkey (*Macaca mulatta*) skin fibroblasts using SKOM retroviruses by Deng and collaborators, whose pluripotency was judged on the base of teratoma formation [82]. A problem of monkeys is that their close phylogenetic relationship with humans still raises ethical concerns and, besides, in most countries there is no easy access to these animals. Aiming to develop a large animal model which is exempt of these problems, Esteban et al. [83] and later on Wu et al. [84] and Ezashi et al. [85] reported the generation of porcine iPSCs using retroviruses or lentiviruses and fibroblasts or bone marrow mesenchymal stem cells from Tibetan mini-pig and farm pig (*Sus scrofa*). Although chimeric animals were not presented, pluripotency was demonstrated by teratoma formation. Notably, reliable teratomas had not been

shown in numerous previous attempts to isolate pig ESCs [86]. Given that the porcine physiology is strikingly similar to humans and their maintenance is easy and relatively inexpensive, the pig stands arguably as the best model for preclinical trials using iPSCs [87]. Difficulties of this model include the mentioned lack of bona fide porcine ESCs with which to establish comparisons, the incomplete sequencing and annotation of the pig genome, and the limited availability of tested reagents, specifically antibodies, that can assist with the characterization of these iPSCs or their derivatives [87]. Besides, in all three studies either the transgenes were not properly silenced [83, 85] or if doxycline was removed the cells differentiated [84], which raises important questions as to whether the reprogramming was indeed complete. Nevertheless, improvement of the current derivation protocols is expected soon and pig iPSCs could play a major role in accelerating the clinical application of human iPSCs.

2 Conclusions

Two major trends have arisen after roughly 4 years of intense iPSC research: the possibility of personalized stem cell therapies using human iPSCs and the creation of in vitro models of human disease. At the current pace of discovery these two types of research may progressively divert and their respective standards could be different. For clinical application iPSC cell lines will have to meet the most stringent criteria of quality and be exempt of transgene insertions. Analyzing the extent of the epigenetic reprogramming in human iPSCs will almost inevitably involve the next generation sequencing technologies. But the analysis of multiple iPSCs is not enough and this will need to be contrasted with ESCs from different sources in order to exclude differences related to the genetic background. This will raise the costs considerably, at least with currently available technologies, and reinforces the idea that further research on human ESCs is important to understand iPSCs, which would surely find many detractors [88]. Altogether this may imply that the long awaited objective of having patient specific pluripotent stem cells is not feasible or at least will take longer than expected. Potential solutions include the creation of a bank of iPSCs matching as many haplotypes as possible, or the production of iPSCs engineered to have low immunogenicity. In both cases the use of fetal sources (e.g., cord blood [54, 55] and umbilical cord matrix mesenchymal cells [89]) should be preferred as these cells do not have the risk of incorporated mutations that is omnipresent in more aged tissues (especially skin cells). By creating iPSC banks, only those iPSC cell lines of the highest quality would be selected, further expanded in the absence of animal products or xenobiotics, and scrupulously tested before clinical trials are approved. Besides, any abnormalities happening afterwards would be immediately noticed and recorded. On the other hand, for modeling genetic diseases in vitro, safety and near perfect epigenetic reprogramming is a priori less of a concern and this parallel field may thus move quicker and face less criticisms. Of course, genetic and epigenetic abnormalities

can also have an impact on the phenotype and be seriously misleading but this problem can be solved by analyzing iPSCs from different affected individuals as well as unaffected controls (ideally from the same family to eliminate the effects of a different genetic background). It is also remarkable that, following the success of iPSC derivation, cells resembling neurons have been produced directly from mouse fibroblasts by overexpressing neural enriched transcription factors [90]. These neurons display electrical activity and can form synapses, but it is unclear yet whether they bear extensive neuron-like epigenetic remodeling and the fibroblast genetic program has been effectively shut down. In the near future we may see other examples of direct transdifferentiation (Fig. 1), and if the transformation is accurate this may end up being more practical than the uphill differentiation into iPSCs and then downhill into specific lineages. In any case, this experiment is a clear indication that iPSC technology is forcing us to think of cell fate as a navigable condition rather than a fixed state, and this directly or indirectly will likely influence how we perceive human physiology and disease.

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Production Process for Stem Cell Based Therapeutic Implants: Expansion of the Production Cell Line and Cultivation of Encapsulated Cells

C. Weber, S. Pohl, R. Poertner, Pablo Pino-Grace, D. Freimark, C. Wallrapp, P. Geigle, and P. Czermak

Abstract Cell based therapy promises the treatment of many diseases like diabetes mellitus, Parkinson disease or stroke. Microencapsulation of the cells protects them against host-vs-graft reactions and thus enables the usage of allogenic cell lines for the manufacturing of cell therapeutic implants. The production process of such implants consists mainly of the three steps *expansion of the cells*, *encapsulation of the cells*, and *cultivation of the encapsulated cells* in order to increase their vitality and thus quality. This chapter deals with the development of fixed-bed bioreactor-based cultivation procedures used in the first and third step of production. The bioreactor system for the expansion of the stem cell line (hMSC-TERT) is based on non-porous glass spheres, which support cell growth and harvesting with high yield and vitality. The cultivation process for the spherical cell based implants leads to an increase of vitality and additionally enables the application of a medium-based differentiation protocol.

Keywords Cell therapy, Mesenchymal stem cells, Encapsulation, Fixed bed bioreactor, Glass carrier

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1 Introduction

Cell based therapy can be defined as the implantation of autologous, allogenic, or xenogenic cells for the replacement of damaged cells or organ functions. Either the implanted cells are able to assume this function by themselves or they stimulate other cells, which then can cause a regeneration of the tissue or organ function. Examples are the treatment of diabetes mellitus with insulin- and GLP-1-secreting cells or Parkinson disease with dopamine-secreting cells as well as the implantation of mesenchymal stem cells for regeneration of heart, cartilage, or bone tissue [1–6].

The implantation of allogenic (or xenogenic) cells demands a protection against host-vs-graft reactions which can be achieved by a suppression of the recipient's immune system or by an encapsulation of the transplanted cells.

Promising, for the treatment of many endocrine or degenerative diseases are microencapsulated stem cells, for example the CellBead® system developed by the CellMed AG (Alzenau, Germany). These cell beads consist of an inner cell containing core which is surrounded by an alginate capsule (Fig. 1). Each cell bead contains about 2,000–3,000 cells in a non-proliferating state. The diameter of the cell containing core bead is about 400 μm and the diameter of the alginate capsule between 500 and 600 μm.

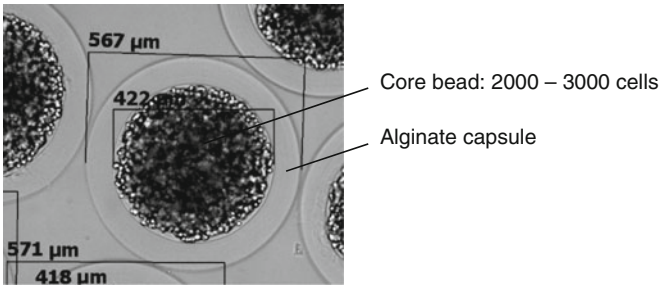

Fig. 1 Light-microscopic image of cell beads

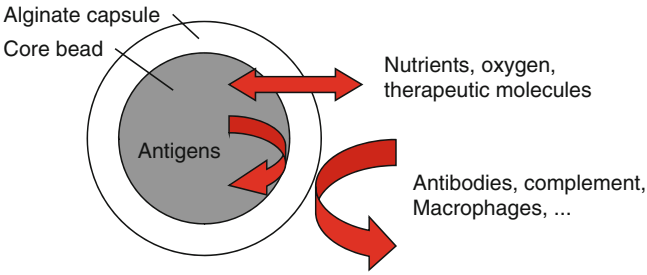

Fig. 2 The alginate capsule of a cell bead acts as a semipermeable membrane

The allogenic production cell line is based on human mesenchymal stem cells, which are transfected with the gene of the catalytic subunit of human telomerase. The telomerase activity counteracts the shortening of the telomeres after each cell cycle, which indicates a transformation of the cells to a permanent cell line [7]. Dependent on the designated use the cells are genetically modified with additional therapeutic genes.

The cell beads can be implanted by injection using a sterile syringe. At the implantation site the cells are provided with nutrients by diffusion through the capsule and in turn release therapeutic molecules. Antigens and components of the host immune system cannot penetrate through the capsule (Fig. 2). The advantages of this therapeutical system are a continuous drug delivery and, due to the local application, high drug concentrations at the point of implantation. Furthermore, a production in stock is possible since a cell line is used.

The implantation of this cell therapeutic system behind the blood-brain barrier makes them suitable for the treatment of, e.g., stroke. Clinical phase 1 studies with GLP-1-transfected cells revealed a reduced loss of neuronal cells in a controlled cortical impact rat model due to the implantation of GLP-1 secreting cell beads [8]. Originally, intestinal cells secrete GLP-1in response to food intake. GLP-1 enhances insulin secretion of beta-cells as well as promoting insulin sensitivity. Since GLP-1 receptors are also expressed throughout the mammalian brain, the implantation of GLP-1 secreting cell beads into the brain may cause neurotrophic and neuroprotective effects.

The production process of cell based implants consists of three steps, whereas this chapter is focused on the first and third production steps (Fig. 3).

Due to the fact that the cells themselves are the product, the requirements of the stem cell expansion process differ from a common mammalian cell culture where, for example, a virus or protein is the product.

The cultivation and harvesting procedure for the expansion of the stem cell based production cell line should be very gentle in order to obtain high yields and vitalities of harvested cells. The used carrier should be non-porous which eases the separation of detached cells from the carrier.

The cell bead cultivation process aims to increase the vitality of the encapsulated cells. A decrease of the cell vitality of the implants may be caused by the harvesting

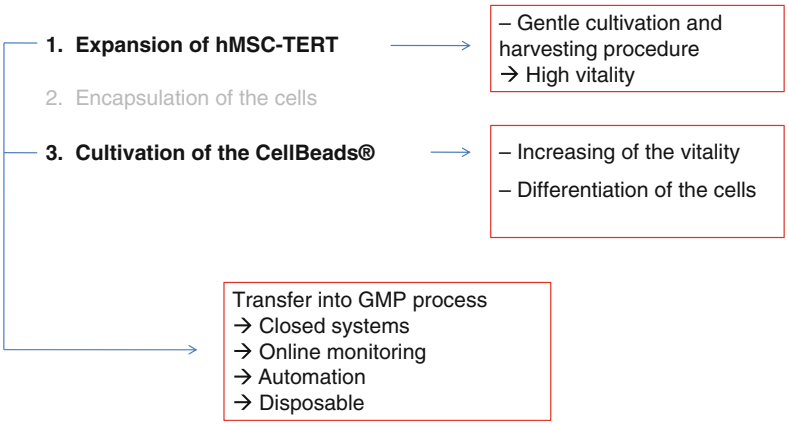

Fig. 3 Steps and requirements of the cell bead production process

and encapsulation procedure. Apoptotic cells will be decomposed during this cultivation.

An optional differentiation of the cell beads may support cell survival at the transplantation place. Furthermore, a differentiation may induce the expression of therapeutic molecules.

Here we introduce two fixed-bed bioreactor systems, one for the expansion of the adherent production cell line and the other for the cultivation of the cell beads. As an example, the application of an adipogenic differentiation protocol was investigated.

Both fixed-bed systems have to be transferred into a GMP-process. Therefore some requirements are addressed to the reactor systems. For maintaining sterility during the process they have to be designed as a closed system with the possibility of online monitoring. Furthermore, automation of the process should be enabled to reduce human error and labor input. A simple design of the reactor system and its peripheral components benefits the manufacturing of disposables, which reduces extensive cleaning procedures and documentation effort. Figure 3 gives an overview about the cell bead production process and its requirements.

2 Expansion of hMSC-TERT

2.1 Reactor System

Many disposable reactor systems used in the cultivation of adherent animal cells like T-flasks, roller bottles, wave reactors, stirred-tank reactors, or spinner flasks are established, but they show more or less drawbacks. T-flasks and their cognate

systems are labor consuming in operation, or intricate in automation. Suspension reactors in combination with a microcarrier have the drawback that a special system for the separation of the enzymatically detached cells from the carrier is necessary. This counteracts a simple design which is demanded for manufacturing as a disposable. Therefore a fixed-bed bioreactor system was developed, which is based on a non-porous carrier. The advantage of a non-porous carrier is that the adherent cells, located on the carrier surface, can be easily flushed out of the reactor with the medium flow after enzymatic detachment. A carrier screening has revealed that non-porous borosilicate glass spheres (BSGS) are most suitable with respect to the growth behavior, cell harvest, and nutrient supply.

Figure 4 shows a prototype of the fixed-bed reactor. It consists of a glass cylinder with a stainless steel lid and bottom plate, which are equipped with hose connectors for the medium inlet and outlet. A funnel shaped insert leads to a nearly uniform inflow. The periphery of the fixed bed consists of some flasks that act as conditioning, collecting, waste, and storage vessels, the tubing, some pinch valves, two peristaltic pumps, single use noninvasive oxygen sensors (PreSens – Precision Sensing GmbH, Regensburg, Germany) for process monitoring, and sterile air filters for adjustment of the vessel pressure. All parts except the valves and the pumps can be designed for production as a disposable, or they are already available as commercial single use items. A further advantage of this system is a

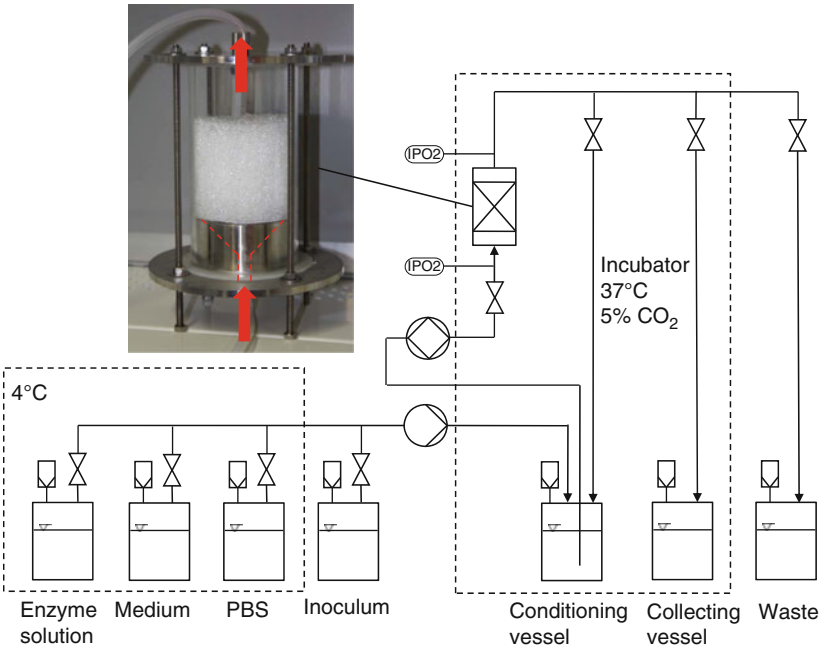

Fig. 4 Fixed-bed reactor system for the expansion of hMSC-TERT

comfortable automation of the process including the inoculation, culturing, and harvesting procedures.

The oxygen supply can be provided by surface aeration or in larger systems with a single use membrane oxygenator. Bubble aeration would have the drawbacks of foam forming when serum containing medium is used.

The reactor system can be operated in a humidified incubator at 37°C and 5% CO_2. Larger scales demand special heating strategies for the fixed bed and the conditioning vessel. Furthermore, a gas mix station is demanded when the medium is aerated by using membrane oxygenators in order to enrich the feed air with CO_2, which is necessary for a pH adjustment of sodium bicarbonate buffered medium.

For determination of growth and consumption kinetics and for scale up calculations a mathematical model was used that is shown here in a simplified form (Fig. 5).

The system is mainly composed of the interacting compartments, the fixed bed, and the conditioning vessel. The concentration c of any nutrient component in the fixed bed depends on time and axial position z and can be expressed by the convection-consumption equation

$$\varepsilon \cdot \frac{\delta c}{\delta t} = -v \cdot \frac{\delta c}{\delta z} - \eta \cdot q \cdot X_{\mathrm{FB}}(t) \tag{1}$$

with the superficial velocity, v, which is defined as the flow velocity in the reactor tube without the packed bed, the cell specific consumption rate q of the regarded nutrient component, the time dependent and volume specific cell density

$$X_{\mathrm{FB}}(t) = X_{\mathrm{FB}}^{0} \cdot \mathrm{e}^{\mu \cdot t} \tag{2}$$

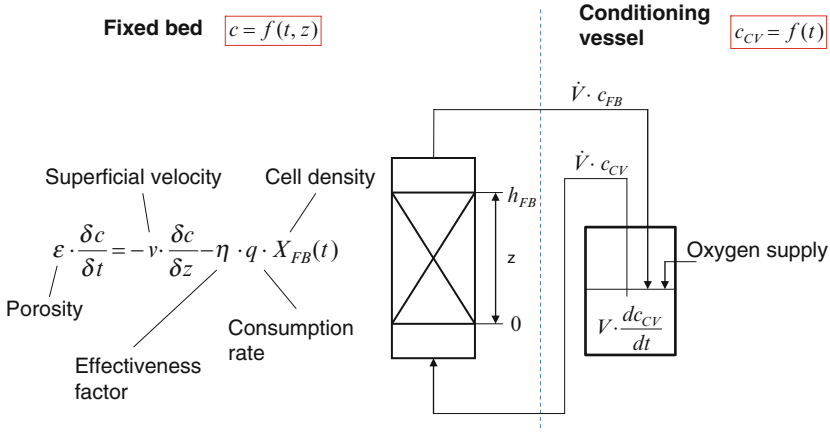

Fig. 5 Simplified illustration of the mass balances of the fixed-bed reactor and its associated conditioning vessel

and the effectiveness factor η, that considers mass transfer resistance [9–14]. The growth rate μ and the glucose consumption rate q_{Glc} can be described by Monod kinetics:

$$\mu = \mu_{\max} \cdot \frac{c_{\mathrm{Glc}}}{c_{\mathrm{Glc}} + k_{M,\mu}} \tag{3}$$

$$q_{\mathrm{Glc}} = q_{\mathrm{Glc,max}} \cdot \frac{c_{\mathrm{Glc}}}{c_{\mathrm{Glc}} + k_{M,q_{\mathrm{Glc}}}} \tag{4}$$

with the Monod constants $k_{M,q_{\mathrm{Glc}}}$ and $k_{M,\mu}$ whereas the oxygen consumption rate q_{Ox} is assumed to be concentration independent:

$$q_{\mathrm{Ox}} = \mathrm{const.} \tag{5}$$

For the concentration c_{CV} in the conditioning vessel only time dependence is assumed. It can be described by balancing the nutrient in- and outflow:

$$V \cdot \frac{\delta c_{\mathrm{CV}}}{\delta t} = -\dot{V} \cdot c_{\mathrm{CV}} + \dot{V} \cdot c_{\mathrm{FB}} \tag{6}$$

with the concentration at the reactor outlet c_{FB}, the medium volume V, and the volume flow $\dot{V}$. In the case of an oxygen balance, 6) has to be extended by the oxygen transfer rate OTR:

$$V \cdot \frac{\delta c_{\mathrm{CV,Ox}}}{\delta t} = -\dot{V} \cdot c_{\mathrm{CV,Ox}} + \dot{V} \cdot c_{\mathrm{FB,Ox}} + \mathrm{OTR} \tag{7}$$

2.2 Expansion of hMSC-TERT on a Laboratory Scale

Fixed-bed cultivations of hMSC-TERT were performed in scales up to a bed volume of 300 cm^3. With these cultivations several process relevant problems could be investigated (Fig. 6).

A *carrier screening* was performed to find a suitable carrier regarding growth and harvesting behavior of the hMSC-TERT.

Inoculation and harvesting procedures were developed, which can be automated and lead to high yields of adhered or detached cells, respectively. Furthermore, the harvesting procedure has to result in a high vitality of the detached cells.

The laboratory scale bioreactor system for the cell expansion has to be *scaled up* to the production scale. Therefore a *maximal superficial velocity has to be defined* in order to avoid negative effects on the cell growth caused by shear stress. Model parameters like growth and consumption rates, which are necessary for scale up calculations, were determined by fitting them to the experimental data.

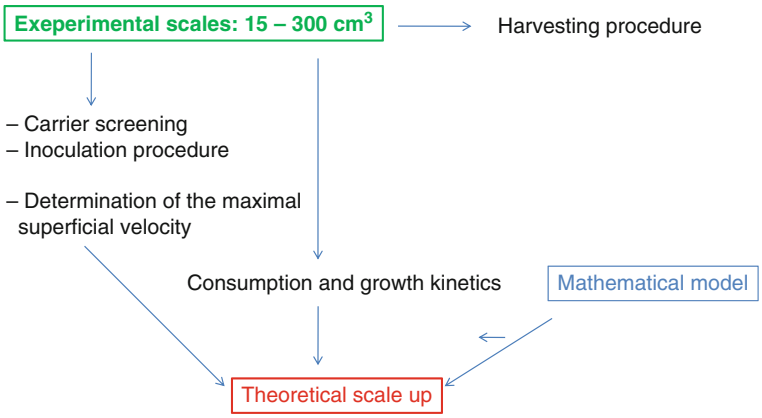

Fig. 6 Overview of performed lab scale experiments for the development of a fixed bed based expansion process for hMSC-TERT

Table 1 Cultivations of hMSC-TERT in 60-cm^3 fixed-bed reactors aimed at finding suitable carrier

Carrier	Sphere diameter d_S (mm)	Growth surface per 60 cm^3 fixed bed A (cm^2)	Medium volume V (ml)	Superficial velocity v (m s^{-1})
BioNoc II™	–	9,600	1,000	1.5×10^{-4}
Borosilicate	1	2,196	500	3.0×10^{-4}
glass	2	1,098	500	3.0×10^{-4}
spheres	3	732	500	3.0×10^{-4}

2.2.1 Carrier Screening

Non-porous BSGS of 1, 2, and 3 mm diameter as well as fibrous macroporous polyethylene terephthalate based carrier (BioNoc II™, Cesco Bioengineering Co, Taichung, Taiwan) were investigated and compared.

Therefore, reactors were filled with 60 cm^3 carrier and inoculated with a cell number corresponding to 5,000 cells per cm^2. During the 4 h inoculation procedure without perfusion of the fixed bed, the reactors were manually turned around 180° after 10, 40, 130, and 240 min in order to return sedimented and non-attached cells into the fixed bed. After the inoculation procedure, the cultivation was started by perfusion of the system with EMEM (Eagle's Minimal Essential Medium) supplemented with 10% fetal calf serum (FCS). Table 1 gives more detail to the cultivations. The final cell numbers at the end of the cultivations were determined by counting of crystal violet stained nuclei after lysis of the cells with citric acid [15].

The results of the cultivations (Fig. 7) are summarized in Table 2. The highest growth rate as well as the highest cell density after 160 h was reached with 2 mm BSGS. The lowest number of attached cells after the inoculation procedure was

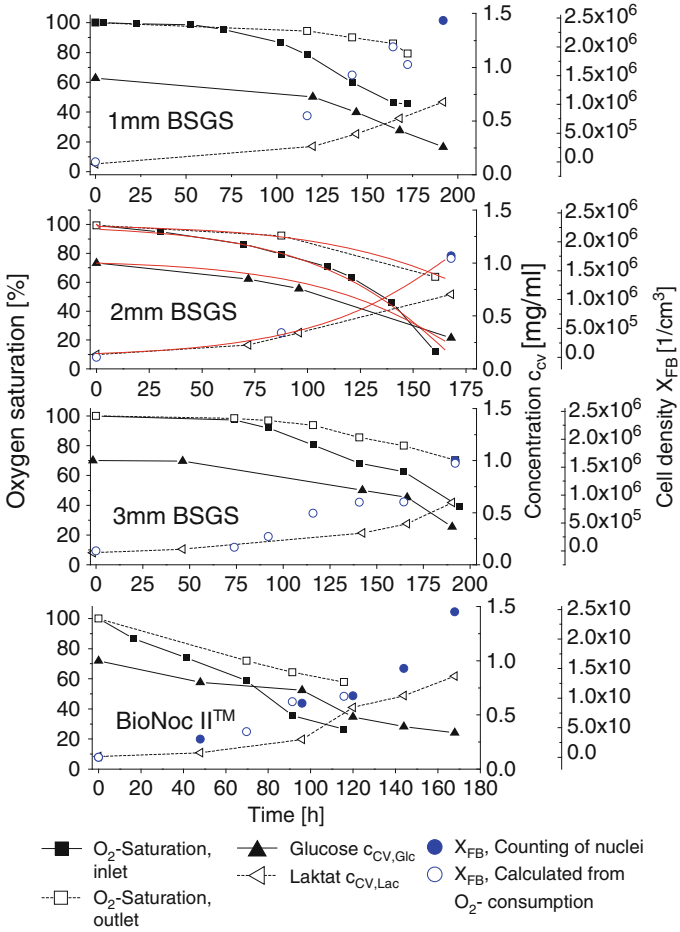

Fig. 7 Cultivations of hMSC-TERT in 60-cm^3 fixed-bed reactors on non-porous borosilicate glass spheres (BSGS) and macroporous BioNoc II™ carrier. *Red curves* (2-mm BSGS) were simulated using the model. *BSGS* borosilicate glass spheres

obtained with BioNoc II™. Channeling was detected by using of BSGS with a diameter of 1 mm, which means a non-optimal nutrient supply and thus limitations of the cells.

Beside the growth behavior, the yield after a harvesting procedure was used for an evaluation of the carrier. Therefore fixed beds, which consisted of 2-mm glass spheres or BioNoc II™, were cyclically perfused with Accutase™ or Trypsin solution for 20 min at a superficial velocity of 1.3×10^{-4} m s^{-1}.

Accutase™ was more effective regarding the yield of detached cells than Trypsin (Fig. 8). The highest yield of 92% was obtained with 2-mm glass spheres and Accutase™.

Table 2 Results of the comparative cultivations of hMSC-TERT in 60-cm^3 fixed-bed reactors on different carrier. The mean growth rates μ_m and the cell densities X_{FB} after the inoculation procedures were obtained by fitting of 2) to the experimental data. BSGS: borosilicate glass spheres

	Units	1 mm BSGS[a]	2 mm BSGS	3 mm BSGS	BioNoc II™
Cultivation time	(h)	192	168	192	168
Mean growth rate μ_m	(1 d^{-1})	0.307 ± 0.062	0.487 ± 0.042	0.372 ± 0.063	0.391 ± 0.034
Cell density at the end of cultivation X_{FB}	(1 cm^{-2})	6.69×10^4	9.64×10^4	1.32×10^5	1.54×10^4
	(1 cm^{-3})	2.45×10^6	1.75×10^6	1.61×10^6	5.63×10^5
Cell density after 160 h X_{FB}	(1 cm^{-2})	$(5.11 \pm 2.20) \times 10^4$	$(9.32 \pm 2.55) \times 10^4$	$(7.92 \pm 3.60) \times 10^4$	$(1.31 \pm 0.29) \times 10^4$
	(1 cm^{-3})	$(1.66 \pm 0.72) \times 10^5$	$(1.71 \pm 0.47) \times 10^5$	$(9.50 \pm 4.31) \times 10^5$	$(2.01 \pm 0.46) \times 10^5$
Cell density after the inoculation procedure X_{FB}^0	(1 cm^{-2})	$(6.58 \pm 2.84) \times 10^3$	$(3.68 \pm 0.99) \times 10^3$	$(6.63 \pm 3.01) \times 10^3$	$(9.68 \pm 2.10) \times 10^2$
	(1 cm^{-3})	$(2.14 \pm 0.92) \times 10^5$	$(6.63 \pm 1.81) \times 10^4$	$(7.96 \pm 3.61) \times 10^4$	$(1.55 \pm 0.34) \times 10^5$
R^2	(–)	0.946	0.998	0.932	0.986

[a]Channeling

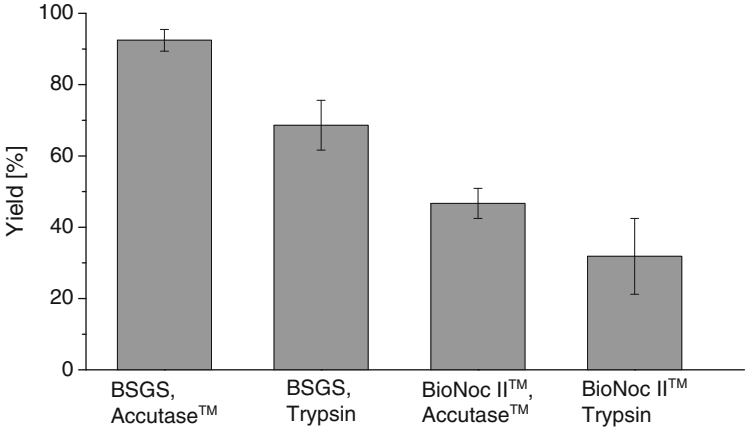

Fig. 8 Preliminary harvesting experiments in fixed-bed reactors containing 2-mm borosilicate glass spheres or BioNoc II™, respectively. The beds were cyclic perfused with enzyme solution for 20 min. The data represent the mean ± standard deviation of four experiments

Considering the previous facts, BSGS with a diameter of 2 mm are most suitable for the cultivation and harvesting procedure of hMSC-TERT. The next steps including the development of automatable inoculation and harvesting procedures as well as the determination of kinetic parameters for scale up calculations were performed with 2-mm BSGS.

2.2.2 Inoculation Procedure

The main problem by inoculation of the fixed bed was that non-adherend cells sedimented and did not get the chance to adhere to the carrier when the system is filled with cell suspension and incubated without perfusion. This yielded a small amount of adhered cells and an axial cell density profile. Therefore, perfusion steps should be included in the inoculation procedure.

Figure 9 shows an inoculation procedure with intermittent perfusion intervals. The yield, which is defined as the ratio of adherend cells to the inoculated cell number, could be increased from 30%, without perfusion, to about 50%, with perfusion.

2.2.3 Determination of the Maximal Superficial Velocity

Due to the non-porosity of the glass carrier, the cells are totally exposed to the shear stress caused by the medium flow, which requires a definition of a maximal superficial velocity. For this purpose, hMSC-TERT were cultured in 25-cm^3 fixed beds at different superficial velocities. The growth rate was used as an evaluation parameter. A reference culture in six-well cell culture plates was performed to get the maximal growth rate at $v = 0$.

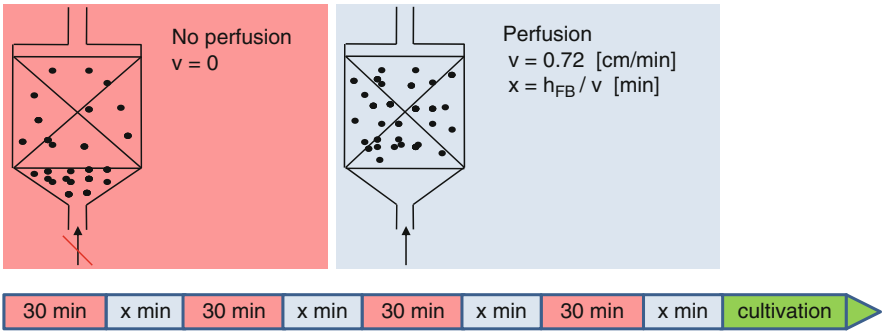

Fig. 9 Scheme of the inoculation procedure. Repeated perfusion steps enhance the number of attached cells and thus the yield of the inoculation procedure

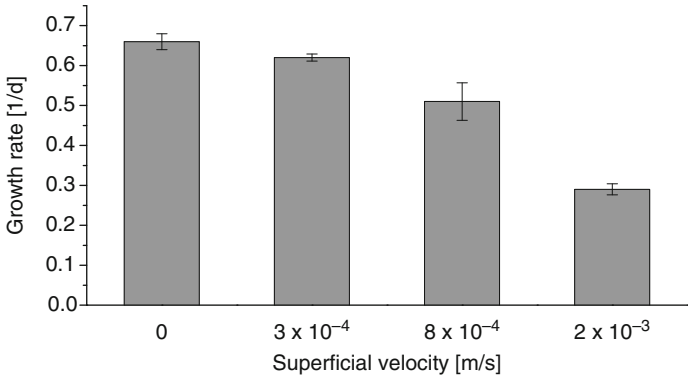

Fig. 10 Growth rates of hMSC-TERT in a fixed bed consisting of non-porous borosilicate glass spheres with a diameter of 2 mm at different superficial velocities. The data represent the mean ± standard deviation of four cultivations

The growth rate starts to decrease at about 3.0×10^{-4} m s^{-1} (Fig. 10). This velocity was used for scale up calculations and experimental determinations of growth and consumption kinetics in fixed-bed reactors.

2.2.4 Cultivation in Different Scales: Consumption and Growth Kinetics

Cultivations were performed in scales from 15 to 300 cm^3. Growth, oxygen, and glucose consumption kinetics were determined by fitting the model parameters to the experimental data. Glutamine wasn't considered since it is an unimportant energy source for hMSC [16]. The modeled curves for a 60-cm^3 scale are exemplarily shown in Fig. 7.

Table 3 Consumption and growth kinetics of hMSC-TERT cultured on 2-mm borosilicate glass spheres in fixed-bed reactors at 15, 60, and 300 cm^3 scales. The data were obtained by fitting the model parameters to the experimental data

Maximal growth rate μ_{max}	0.55 – 0.69	(1 day^{-1})
Monod constant $k_{M,\mu}$	0.135 – 0.160	(mg mL^{-1})
Maximal glucose consumption rate $q_{Glc,max}$	(8.0 – 11.8) × 10^{-8}	(mg h^{-1} $cell^{-1}$)
Monod constant $k_{M,q_{Glc}}$	0.10 – 0.16	(mg mL^{-1})
Oxygen consumption rate q_{Ox}	(0.98 – 2.14) × 10^{-8}	(mg h^{-1} $cell^{-1}$)

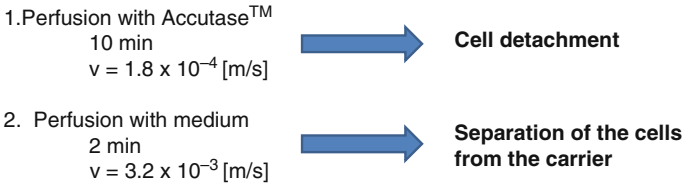

Fig. 11 Harvesting of hMSC-TERT in fixed-bed reactors based on non-porous borosilicate glass spheres

Table 3 shows the fitted model parameters. The growth and consumption rates are comparable to those reported in the literature. Glucose consumption and growth rates of human mesenchymal stem cells are reported to be (0.23 – 1.22) × 10^{-7} mg h^{-1} $cell^{-1}$ [17, 18] and 0.33 – 0.94 1 d^{-1} [15, 16, 19–22], respectively. Oxygen consumption rates of (1.22 – 3.20) × 10^{-8} mg h^{-1} $cell^{-1}$ are reported for various human cell types [23–27].

Most important for scale up considerations is the oxygen consumption rate.

2.2.5 Harvesting Procedure

A drawback of the preliminary harvesting procedure used for the carrier screening was that, due to the cyclic perfusion of the enzyme solution, already detached cell were repeatedly passed through the reactor system. This caused shear stress which led to a decrease in vitality to values below 90% (data not shown). Therefore, a harvesting procedure was developed in which the cell suspension will be directly connected to the collecting vessel (Fig. 11).

In the first step, cells become detached by perfusion of the fixed bed for 10 min with Accutase™ solution at a superficial velocity of 1.8 × 10^{-4} m s^{-1}. After this the cells get flushed out by perfusion with medium at a superficial velocity of 3.2 × 10^{-3} m s^{-1} for 2 min. This harvesting procedure resulted in a yield of detached and separated cells of approximately 82% and a vitality of about 96%.

The advantages of this procedure are the comfortable automation and separation of the detached cells without any further system components or process steps.

2.3 Theoretical Scale Up of the hMSC-TERT Expansion Process

For scale up calculations of the fixed bed, it is mainly the dissolved oxygen concentration that has to be considered, since it is magnitudes lower than other limiting medium components such as glucose. The oxygen concentration or saturation, respectively, decreases in axial direction that demands a definition of a maximal fixed-bed height h_{FB} (Fig.12).

The maximal fixed-bed height depends, assuming a 100% air saturated inflow concentration and a constant inflow velocity (3.0×10^{-4} m s^{-1}), on the maximal cell density and the minimal oxygen concentration in the fixed bed, which can be found at the outflow region. This maximal bed height can be calculated by using the previously described model:

$$h_{FB} = f(X_{FB}, c_{Ox,out}) \tag{8}$$

Using the maximal bed height, a calculation of the maximal volume V_{FB} of a single fixed bed as a function of the thickness ratio TR and with it a calculation of the needed number n_{FB} of parallel operated fixed-bed reactors for the cultivation of a certain target cell number N_X is possible:

$$V_{FB} = h_{FB} \cdot \pi \cdot \left(\frac{h_{FB}}{TR \cdot 2}\right)^2 \tag{9}$$

$$n_{FB} = \frac{N_X}{X_{FB} \cdot V_{FB}} \tag{10}$$

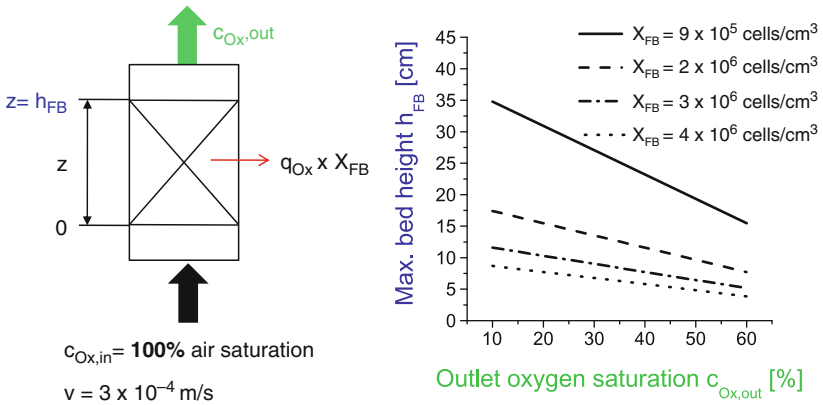

Fig. 12 Dependency of the bed height on the outlet oxygen saturation and target cell density calculated using the model of the cultivation process and the maximal oxygen consumption rate (Table 3)

Table 4 Numbers and volumes of parallel operated fixed beds needed for the cultivation of 2×10^{10} cells as a function of thickness ratio, target cell density, and outlet oxygen saturation

Target cell density (cm^{-3})	Thickness ratio = 1		Thickness ratio = 2		Total fixed-bed volume (L)
	Fixed-bed volume V_{FB} (L)	Number of fixed beds n_{FB}	Fixed-bed volume V_{FB} (L)	Number of fixed beds n_{FB}	
Outlet oxygen saturation: 20%					
1×10^6	**20**	**1.1**	**5.2**	**4.2**	21.9
2×10^6	**2.3**	**4.8**	0.6	19.1	10.9
4×10^6	0.3	18.3	0.07	73.1	5.5
Outlet oxygen saturation: 30%					
1×10^6	**13.4**	**1.6**	3.4	6.5	21.9
2×10^6	1.5	7.3	0.4	29.0	10.9
4×10^6	0.2	27.8	0.05	111.4	5.5

The reactor system was scaled exemplarily for the cultivation of a target cell number of 20 billion cells that is sufficient for approximately 200 single doses of cell beads with a volume of about 5 mL per dose (Table 4).

The volume of a single fixed bed decreases with increasing cell number, thickness ratio, and outlet oxygen saturation. Small reactor volumes means a large number of parallel operated fixed-bed reactors, but this is very intricate and non-practical regarding the handling and operation of the bioreactor system. Therefore a reduction of the target cell density, as well as a reduction of the thickness ratio and the outlet oxygen saturation, is recommended. As a result of this, small numbers of parallel operated reactors which can be handled and operated more easily are obtained. The oxygen outlet concentration, of course, can only be decreased to an uncritical value.

3 Cultivation of Encapsulated Cells

3.1 The Reactor System

The cell bead cultivation system is based on single use plastic syringes in which the cell beads themselves form the bed. The original piston is replaced by a custom made insert, which enables perfusion of the fixed bed (Fig. 13).

Beside the cultivation on a single dose level, the advantages of this bioreactor system are a cryopreservation of the cell beads by direct freezing of the syringe and post-thaw an implantation of the cell beads using this syringe. This avoids contamination risky transfer steps. For this purpose, the insert can be designed in such a manner that it can act as the original syringe piston.

The reactor periphery is similar to that of the hMSC-TERT expansion system. Furthermore, the previously described mathematical model can be used as well for the simulation of the cell bead cultivation process.

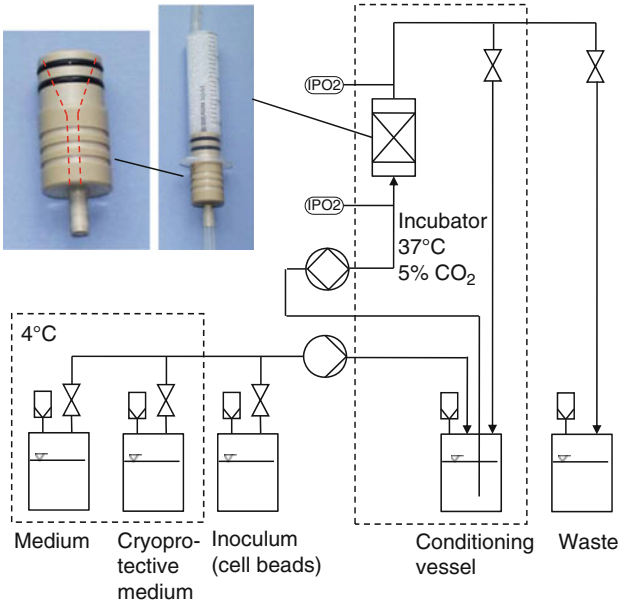

Fig. 13 System for the cultivation of cell beads in syringe based fixed reactors

Table 5 Composition of media for the adipogenic cultivation of cell beads [28]

Induction medium	Maintenance medium
DMEM + 10% FCS	DMEM + 10% FCS
100 U mL^{-1} penicillin	100 U mL^{-1} penicillin
0.1 mg mL^{-1} streptomycin	0.1 mg mL^{-1} streptomycin
0.01 mg mL^{-1} insulin	0.01 mg mL^{-1} insulin
0.5 mM 3-isobutyl-1-methyl-xanthin	–
0.2 mM indomethacin	–
1 μM dexamethason	–

3.2 Cultivation of Encapsulated Cells

Cultivations of cell beads were exemplarily performed under adipogenic conditions on a 1-cm^3 scale (data not shown). Induction medium was applied for 3 days followed by 4 days cultivation with maintenance medium (Table 5). This cycle was repeated three times [14]. Medium, 50 mL per cycle, was perfused at a superficial velocity of 1.28×10^{-4} m s^{-1}, that is below the fluidization point. Reference cultures were performed in 25-cm^2 T-flasks using the same protocol.

Vitality was determined after 0, 100, 200, and 500 h with the Trypan blue exclusion method after lysis of the cell beads using EDTA [28].

The vitality increased with advancing cultivation time, whereas the cell number decreased (Fig. 14). This is explainable by decomposition of apoptotic cells. Apoptosis may be triggered by the harvesting or the encapsulation process, or

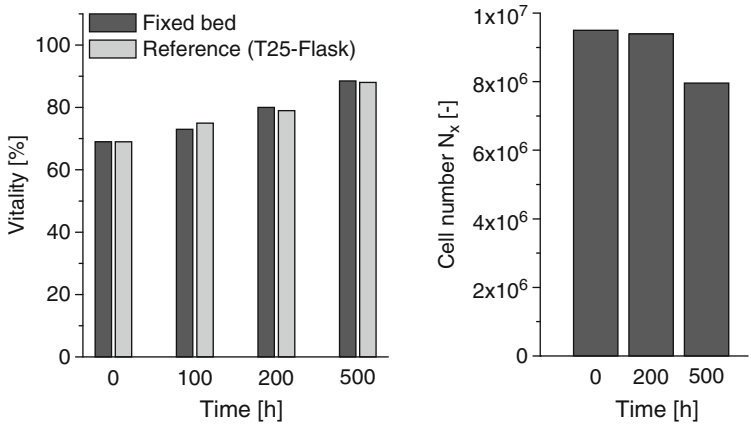

Fig. 14 Time dependent vitality and cell number of cell beads cultured in fixed-bed reactors and in T-flasks (reference)

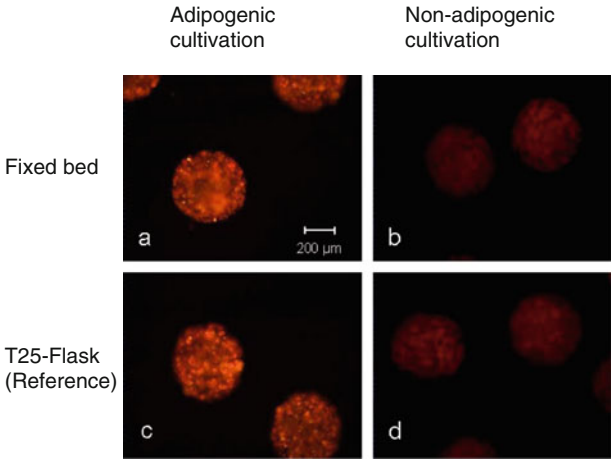

Fig. 15 Nile red staining of cell beads which were cultured under adipogenic and non-adipogenic conditions in fixed-bed reactors or T25-flasks

dominant in this case, by the cryopreservation of the cell beads prior to the cultivation. It could be shown that the vitality, and thus the quality of the beads, is gradable during the cultivation process.

As an example, an adipogenic differentiation protocol was applied, whereby the differentiation to adipocytes was verified by staining with the lipophilic fluorescence dye Nile red (Fig. 15) [28]. Adipogenic cultured cell beads showed higher fluorescence intensity and thus are interpreted to be differentiated to adipocytes. No differences between the fixed-bed culture and the reference culture in T-flasks are detectable.

Table 6 shows the kinetics obtained by fitting the model parameter to the experimental data which were used for a theoretical scale up of the system.

Table 6 Consumption kinetics of encapsulated hMSC-TERT (cell beads) which were obtained by fitting of model parameters to the experimental data of the adipogenic cultivation in a 1-cm^3 fixed-bed scale (Fig. 14)

Growth rate μ_{max}	0	(1 day^{-1})
Maximal glucose consumption rate $q_{Glc,max}$	(7.3 – 9.4) × 10^{-8}	(mg h^{-1} cell^{-1})
Monod constant $k_{M,q_{Glc}}$	0.06	(mg mL^{-1})
Oxygen consumption rate q_{Ox}	5.5 × 10^{-9}	(mg h^{-1} cell^{-1})

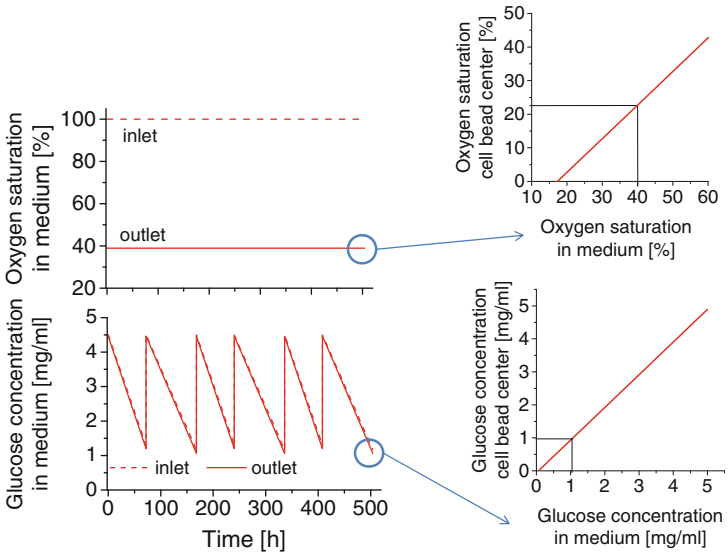

Fig. 16 Simulated adipogenic cultivation of 200 single doses (5 cm^3) of cell beads as well as the glucose and oxygen profile at the cell bead center. Medium volume per cycle: 40 L, superficial velocity: 2.5 × 10^{-4} m s^{-1}

3.3 *Theoretical Scale Up of the Cell Bead Cultivation Process*

A calculational scale up was carried out for a cultivation of 200 single doses of cell beads each of 5 mL (Fig. 16). The inlet oxygen concentration was assumed to be air saturated. This can be realized, for example, by using membrane oxygenators.

For a calculation of the oxygen or glucose concentration profile in the cell bead, the following diffusion and diffusion-reaction equations were used:

$$D_{CC} \cdot \left(\frac{d^2 c_{CC}}{dr^2} + \frac{2}{r} \cdot \frac{dc_{CC}}{dr} \right) = q \cdot X_{CC} \tag{11}$$

$$D_{AC} \cdot \left(\frac{d^2 c_{AC}}{dr^2} + \frac{2}{r} \cdot \frac{dc_{AC}}{dr} \right) = 0 \tag{12}$$

with the concentration in the cell containing core bead c_{CC} as well as in the cell free alginate capsule c_{AC}, the effective diffusion coefficient in the core bead D_{CC} and alginate capsule D_{AC}, and the cell density of the core bead X_{CC}.

It could be shown that an oxygen saturation in the medium of 40% leads to an oxygen saturation at the center of approximately 22% (Fig. 16). The differences in glucose between the medium and the cell bead center is negligible. Thus, it can be assumed that no limitations of the cells at the center of a cell bead are expectable.

3.4 Conclusion and Outlook

Two fixed-bed reactor systems for the production of stem cell based therapeutic implants were introduced. One system was developed for the expansion of the production cell line (hMSC-TERT) and a second for the cultivation of encapsulated cells in order to increase their vitality and thus the quality of the implants.

The fixed-bed system for the expansion of the production cell line is based on non-porous BSGS. Cells can be cultured and harvested with high yield and vitality. The separation of the cells from the carrier can easily be performed by flushing them out with the medium flow. This saves additional process steps.

The fixed-bed system for the cultivation of encapsulated cells is based on commercially available syringes in which the cell beads represent the bed. The advantage of this system is that it can be used as an implantation tool after the cultivation procedure. It could be shown that the vitality is gradable by the cultivation process. Furthermore, the application of an adipogenic differentiation protocol could be demonstrated.

Both systems can be automated and produced as disposable items due to their simple design.

The next steps will concern the development of a GMP-conform cryopreservation procedure for the cell beads and the implementation of the cultivation systems to the overall GMP-process of cell bead production.

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Cartilage Engineering from Mesenchymal Stem Cells

C. Goepfert, A. Slobodianski, A.F. Schilling, P. Adamietz, and R. Pörtner

Abstract Mesenchymal progenitor cells known as multipotent mesenchymal stromal cells or mesenchymal stem cells (MSC) have been isolated from various tissues. Since they are able to differentiate along the mesenchymal lineages of cartilage and bone, they are regarded as promising sources for the treatment of skeletal defects. Tissue regeneration in the adult organism and in vitro engineering of tissues is hypothesized to follow the principles of embryogenesis. The embryonic development of the skeleton has been studied extensively with respect to the regulatory mechanisms governing morphogenesis, differentiation, and tissue formation. Various concepts have been designed for engineering tissues in vitro based on these developmental principles, most of them involving regulatory molecules such as growth factors or cytokines known to be the key regulators in developmental processes. Growth factors most commonly used for in vitro cultivation of cartilage tissue belong to the fibroblast growth factor (FGF) family, the transforming growth factor-beta (TGF-β) super-family, and the insulin-like growth factor (IGF) family. In this chapter, in vivo actions of members of these growth factors described in the literature are compared with in vitro concepts of cartilage engineering making use of these growth factors.

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Keywords Bone morphogenetic protein (BMP), Cartilage, Chondrocytes, Differentiation, Fibroblast growth factor (FGF), Growth factors, Indian hedgehog (Ihh), Insulin like growth factor (IGF), Mesenchymal stem cells (MSC), Multipotent mesenchymal stromal cells (MSC), PTH related peptide (PTHrP), Sonic hedgehog (Shh), Transforming growth factor-beta (TGF)

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Abbreviations

AER	Apical ectodermal ridge
BMP	Bone morphogenetic protein
ECM	Extracellular matrix
FGF	Fibroblast growth factor
FGFR	FGF receptor
GF	Growth factor
HA	Hyaluronic acid
IGF	Insulin-like growth factor
IGFBP	IGF binding protein
IGFR	IGF receptor
Ihh	Indian hedgehog

MSC	Mesenchymal stem cells
OA	Osteoarthritis
PTHrP	PTH related peptide
RA	Rheumatoid arthritis
Shh	Sonic hedgehog
TGF-β	Transforming growth factor-β
ZPA	Zone of polarizing activity

1 Introduction

1.1 The Term "Mesenchymal Stem Cells"

For cartilage regeneration in vitro and in vivo, various strategies have been pursued regarding the appropriate cell source, chemical and physical factors, and culture conditions. It is widely agreed that tissue regeneration from autologous cells can be achieved taking advantage of the natural course and progression of embryonic development [1–4]. Therefore, autologous mesenchymal stem cells (MSCs) are extensively investigated for their ability to regenerate articular cartilage tissue in situ or in vitro.

Tissues forming the skeleton of the limbs originate from stem cells of the lateral plate and the somitic mesenchyme [2]. These embryonal MSCs undergo a series of differentiation steps, finally producing differentiated skeletal tissues such as bone and cartilage. In the adult organism, there are a limited number of mesenchymal progenitor cells residing in the bone marrow which give rise to the repair of damaged tissue, for instance bone, and which can easily be obtained by marrow aspiration and selected by their ability to adhere to culture vessels. To date, it is not clear whether they represent cells remaining from the embryonic mesenchyme or whether they are a heterogenous population of mesenchymal precursor cells [4, 5], possibly originating from the invading blood vessels populating the newly formed bone marrow space during endochondral ossification [6].

First evidence for a precursor pool within the bone marrow was given by Friedenstein et al. [7], who described ectopic osteogenic differentiation originating from whole bone marrow. Based on these results, they hypothesized a population of cells occurring in the bone marrow which are able to differentiate along the osteogenic lineage. These cells were characterized in vitro as colony forming fibroblastic cells (CFU-f), isolated by their adherence to culture vessels [8]. MSC were hypothesized as progenitor cells of mesenchymal tissues residing in the bone marrow and periosteum, persisting throughout lifetime as a pool for tissue regeneration, and which might be isolated, expanded, and used for autologous treatment of damaged tissues [9]. In 1999, Pittenger et al. [10] demonstrated the ability of clonally expanded human bone marrow cells to differentiate towards the osteogenic, chondrogenic, and adipogenic lineages and thereby making it possible to develop treatments with human

autologous cells for the repair of mesenchymal tissues such as bone, cartilage and adipose tissue. Since that time, these so-called MSCs have been isolated from various tissues including adipose tissue [11], muscle and brain [12], bone [13], synovium [14, 15], umbilical cord [16], and blood [17]. Cells with even higher differentiation potential have been isolated from cord blood [18, 19] and from bone marrow [20].

In order to characterize the MSCs, several markers have been described, but none of them proved to be unique and exclusively present on MSCs. Enrichment of MSCs has been carried out using the Stro-1 monoclonal antibody [21, 22]. The resulting cell population was shown to be able to differentiate into the mesenchymal lineages of osteoblasts, chondrocytes, adipocytes, and stromal cells supporting hematopoiesis [23]. Other antigens specific for undifferentiated precursor cells and absent on differentiated cells have been identified using the monoclonal antibodies SH-2 (endoglin, co-receptor for TGF-β3) [24], SH-3, and SH-4, respectively [25]. But there are no unique surface antigens so far defining "the" MSC.

Clonally derived MSCs have been extensively analyzed for their ability to proliferate in vitro, retaining their multi-lineage differentiation potential upon prolonged cultivation. It was shown that these clonally derived cells lost their multi-lineage potential upon extended cultivation in vitro and thus behave like plastic progenitor cells rather than stem cells [5, 26–28]. On the other hand, Jiang et al. [20] were able to describe a subset of pluripotent cells in the mouse and the rat bone marrow, virtually proliferating for up to 60 passages without losing their characteristic growth rates and their potential even to transdifferentiate into the endodermal and ectodermal lineages displaying some features of hepatocytes or neuronal cell types [20, 29]. Thus it is likely that MSCs are a heterogenous population of rare cells in the bone marrow compartment and other tissues, displaying various stages of predifferentiation. Due to the heterogeneity of the cell preparations commonly referred to as MSCs, Dominici et al. defined mesenchymal stem cells as plastic adherent cells positive for CD105, CD73, CD90 lacking the expression of hematopoetic markers CD45, CD34, CD14 or CD11, CD79 alpha or CD19 and HLA-DR surface markers [30]. Finally, according to this definition, MSCs must be shown to differentiate into osteoblasts, adipocytes and chondrocytes in vitro. According to Horwitz et al. [31], fibroblast-like plastic adherent mesenchymal progenitor cells isolated from various tissues are termed multipotent mesenchymal stromal cells (also referred to as MSCs).

Recently, it has been proposed to define "stemness" as a state of cells which are able to differentiate into various cell types rather than the cells themselves [32]. According to this view, plasticity would be the most prominent characteristic of stem cells whereas self-renewal and hierarchical differentiation are regarded as subordinate features of these cell populations. Therefore, dedifferentiated cells capable of differentiating into more than their original cell type could also be regarded as stem cells. Since in vitro-expanded and thus dedifferentiated articular chondrocytes were shown to differentiate similarly to MSCs along the mesenchymal lineages into osteoblasts, chondrocytes, including hypertrophic chondrocytes, and adipocytes [33, 34], the term "secondary progenitor cells" could be appropriate for dedifferentiated chondrocytes [35].

1.2 Concepts for Cartilage Cultivation In Vitro

The regeneration of damaged tissues in the adult organism as well as in vitro engineering of tissues is hypothesized to follow the principles of embryogenesis [1–4]. Therefore it is assumed that cartilage formation by means of cell therapy or tissue engineering is bound to recapitulate, at least in several aspects, the stages of in vivo development. The development of the appendicular skeleton is initiated by migration of the early mesenchymal progenitors of the skeletal tissue towards the prospective limb regions and by proliferation of these undifferentiated progenitors. The accumulation of high cell densities is the prerequisite of cell condensation as the key event of cartilage tissue formation. Thus, the factors governing the migration and proliferation of the undifferentiated mesenchymal cells hold potential for the expansion of undifferentiated precursor cells in vitro.

The morphogenesis of skeletal elements requires precartilage condensation leading to chondrogenic differentiation and thus to the formation of cartilaginous models of the skeletal elements. During chondrogenic differentiation, the cells adopt a rounded morphology and start to synthesize cartilage specific matrix molecules. Further shaping of the skeletal primordia involves the separation of the digital rows by apoptosis of the cells within the interdigital mesenchyme. The concomitant formation of the joints requires dedifferentiation of the cells in the prospective joint regions transiently leading to high cell densities and finally to apoptotic cell death and the development of the joint cavity.

Since the precursor cells are usually expanded in 2D systems in vitro where they display a flattened spindle-shaped morphology, 3D culture systems are supposed to be appropriate for chondrogenic differentiation. The naturally occurring events are mimicked by cell–cell-contacts established in high density pellet culture [36] by cultivation in hydrogels [37–41] or 3D matrices of biocompatible materials [42, 43]. A comparison between the in vivo stages of cartilage development and the strategies used for transferring these stages into in vitro concepts and the inherent challenges is given in Table 1.

Usually these culture systems taken alone are not sufficient to induce chondrogenesis of precursor cells. Therefore, growth factors are included into the culture medium and combined with physical factors such as reduction of oxygen supply

Table 1 Strategies for transferring in vivo development into in vitro concepts

In vivo development of cartilage tissue	In vitro cultivation of cartilage tissue
Migration and proliferation	Expansion of MSC in vitro (2D cultivation)
Prechondrogenic condensation	3D aggregate culture imitating cellular condensation
Chondrogenic differentiation	Cultivation in 3D biomaterials mimicking the 3D structure of the ECM
Transient growth plate cartilage: hypertrophy and apoptosis	
Permanent articular cartilage: no hypertrophy, no apoptosis	Prevention of hypertrophy and apoptosis in articular cartilage regeneration

which occurs naturally by exclusion of the blood vessels from forming limb buds. Chondrogenic differentiation in vitro is achieved after cultivation for several weeks resulting in the formation of collagen type II and proteoglycans along with other cartilage specific tissue components.

In vivo, the articular cartilage is maintained in a stage in which progression towards hypertrophy naturally occurring in the growth plate is prevented by defined mechanisms. In the growth plate, hypertrophy and calcification of the cartilage tissue precede vascular invasion, finally leading to tissue replacement by bone. The inactivation of the differentiation program towards hypertrophy is particularly important for in vitro cultivation of cartilage tissue when MSCs are used as the cell source since terminally differentiated cells will undergo apoptosis. Unlike MSCs, articular chondrocytes usually do not undergo lineage progression upon extended cultivation [44] or when implanted into ectopic sites [45]. Maintenance of the phenotype of articular cartilage therefore comprises the inactivation of the developmental pathway leading to terminal differentiation, and a switch to sustained maintenance of functional extracellular matrix. For this reason, the appropriate stage of in vitro cultivated cartilage tissue needs to be carefully evaluated regarding the state of cellular phenotype of chondrocytes and the characteristics of the extracellular matrix formed.

In this overview, regimes of growth factor treatments currently applied in cartilage tissue engineering in vitro are compared to stage specific actions of growth factors in vivo during cartilage differentiation. Since growth factors of the fibroblast growth factor (FGF)-family, transforming growth factor-β (TGF-β) superfamily and the insulin-like growth factor (IGF) family are widely used in cartilage engineering in vitro, the overview will be narrowed to these major groups of growth factors. An overview of in vivo actions with in vitro applications of growth factors is given in Table 2.

2 Development of Cartilage Tissue In Vivo

The development of the skeleton in vivo is a multistep process tightly regulated regarding the temporal and spatial distribution of the appropriate signals. Morphogenesis is evoked by gradients of specific signaling molecules along the axes of the developing limbs. Following steps are triggered by the achievement of distinct differentiation stages of the cells and by stage specific extracellular matrix synthesis. Regulatory mechanisms such as feedback terminate a distinct developmental stage and allow for further differentiation. Thus, tissue development depends on the differentiation stage of the cells as well as on signals provided by paracrine, autocrine or systemic factors, and the extracellular matrix (Fig. 1).

Taking into account the multi-stage process leading to functional tissues in vivo, histogenesis in vitro also needs to be tightly regulated regarding the time sequence of induction and the resulting consecutive stages of extracellular matrix synthesis (Fig. 2).

Table 2 Occurrence and actions of growth factors in developing and mature cartilage, diseased cartilage tissue, and applications in cartilage cultivation in vitro

Growth factor/ receptor	Role in the development of cartilage tissue in vivo	Role in adult cartilage tissue	Role in cartilage lesions or in articular cartilage disease	In vitro applications and functions
FGF family				
FGF-2	Present in the AER	Bound to heparan sulfate in the ECM, possible role in mechanotransduction [97–99]	Recruitment of MSCs to cartilage defects	Stimulation of proliferation of MSC in vitro [146]
	Stimulation of Tenascin synthesis in mesenchymal condensations [147]	–	Induction of MMP-13 [101] and noggin [104]	Maintenance of differentiation potential of MSCs [126, 127]
	Stimulation of tenascin and syndecan synthesis in condensations limiting size of condensations [64]	–	Increased production in RA [148]	Increased integrin alpha10-expression on cultured MSCs in vitro [128]
	Activation by endothelial proteases in endochondral ossification [149]	–	–	Maintenance of differentiation potential of mammalian chondrocytes in vitro [123]
	–	–	–	"Dedifferentiation" of chondrocytes [33]
FGF-4	Occurrence in the AER, chemoattractant for limb bud mesenchymal cells [46]	–	–	–
	Occurrence in the ZPA, role in AP axis formation, controls the formation of gap junctions [150, 151]	–	–	–
FGF-8	Expression in the AER, maintenance of fgf-10 expression in the underlying mesenchyme [47, 75, 77]	–	–	–

(*continued*)

Table 2 (continued)

Growth factor/ receptor	Role in the development of cartilage tissue in vivo	Role in adult cartilage tissue	Role in cartilage lesions or in articular cartilage disease	In vitro applications and functions
FGF-10	Lateral plate mesenchyme:	–	–	–
	Proliferation, migration [73, 77]	–	–	–
	Expression in blastemas of regenerating limbs of amphibia [78]	–	–	–
FGF-18	Role in endochondral ossification, coordination of chondrogenesis and osteogenesis [152, 153]	–	–	–
	Signaling via FGFR3, promotion of chondrogenesis [154]	–	–	–
FGF receptors				
FGFRI	Mesenchymal cells of the limb bud prior to condensation [155, 156]	–	–	Expression in proliferating cells of mesenchymal lineage in vitro [157]
	Absence of FGFR1 causes apoptosis in limb buds [158]	–	–	Expression prior to differentiation [130, 131]
	–	–	–	Expression in hypertrophic cultures of MSCs [130, 131]
FGFRII	Mesenchymal condensations [156], essential for limb outgrowth [74]	Adult articular cartilage [130]	–	Expression in condensations of mesenchymal micromass cultures [157]
	Expression in limb bud ectodermal epithelium [155]	–	–	Expression during condensation in vitro [131]
	Ectodermal FGF-2 and FGF-8 inhibit chondrogenesis through FGFRII signaling [159]	–	–	Down regulation during differentiation in vitro [130]
	In prechondrocytic cells prior to condensation [155]	–	–	–

FGFRIII	Growth plate chondrocytes [155, 160]	–	–	Prior to calcification of mesenchymal micromass cultures [157]
TGF-β superfamily				
TGF-β1	Expression in condensations, stimulates synthesis of fibronectin Tenascin, N-CAM, N-cadherin [50, 64, 65]	Present in adult cartilage, bound to cartilage matrix [107]	Induction of OA-like changes upon overexpression [111]	Induction of chondrogenesis of bone marrow MSC [133] and synovium derived MSC [137]
	Fn positive areas precede chondrogenic differentiation [86]	Stimulation of fibronectin synthesis in cartilage explants [161]	Stimulation of proteoglycan synthesis by OA chondrocytes [162, 163]	Inhibitory effect on primary chondrocytes [164, 165] Inhibition of terminal differentiation of epiphyseal chondrocytes [166]
	TGF binding domain in Procollagen type IIA [167]	–	Upregulation of TGF-β expression in OA [168]	–
	Storage in growth plate matrix bound to LTBP, activation by matrix vesicles [107, 108]	–	–	–
	Stimulation of PTHrP synthesis in epiphyseal chondrocytes [169]	–	–	–
	Proliferation of undifferentiated mesenchymal cells [79]	–	–	–
TGF-β2	Condensations	Present in adult cartilage	Proliferation	Induction of matrix synthesis, Endogenous synthesis by ATDC-5 aggregate cultures [170]
	–	–	–	Inhibitory effect on proliferation of MSCs [146]

(*continued*)

Table 2 (continued)

Growth factor/ receptor	Role in the development of cartilage tissue in vivo	Role in adult cartilage tissue	Role in cartilage lesions or in articular cartilage disease	In vitro applications and functions
TGF-β3	–	–	Proliferation, stimulation of matrix synthesis	Chondrogenic differentiation of MSCs [10, 36]
	–	–	Induction by IL-1 [112]	–
BMP (general)	Negative regulators of the AER [81]	–	–	ATDC5 cell line: stage specific expression of BMP-2, -4, -6, -7 [171]
	Feedback inhibition by Noggin [172]	–	–	–
	BMP signaling required for maintenance of differentiated phenotype, cell proliferation and hypertrophy [173]	–	–	–
	Inhibition of BMP signaling by noggin inhibits condensation and differentiation [91]	–	–	–
	BMPs act as heterodimers [82]	–	–	–
	Co-localization of BMPs [174]	–	–	–
	Feedback inhibition of BMP signaling by noggin [175]	–	–	–
BMP-2	Expression in the AER and ZPA	–	–	Stimulation of N-cadherin synthesis and chondrogenesis in C3H10T1/2 cells [176]
	Induction of collagen type X synthesis and alkaline phosphatase expression in growth plate chondrocytes [177]	–	–	Induction of chondrogenesis of bone marrow MSC [178] and synovium derived MSC [15] and synovial explants [137]

BMP-4	Induction of collagen type X synthesis and alkaline phosphatase expression in growth plate chondrocytes [177]	–	–	–
BMP-6	Transient expression in prehypertrophic chondrocytes [179, 180]	–	–	Chondrogenic differentiation of MSCs, synthesis by prehypertrophic cells in vitro [171]
BMP-7 (OP-1)	Expression during chondrogenesis	Synovial fluid, cartilage tissue	Reduced expression in aged cartilage tissue [113]	Synthesis of collagen type II, aggrecan, GAG
	Induction of collagen type X synthesis and alkaline phosphatase expression in growth plate chondrocytes [177]	–	–	Induction of chondrogenesis in ATDC5 cell line [181]
	–	–	–	Induction of chondrogenesis of synovial explants [137]
	–	–	–	Redifferentiation of articular chondrocytes [182, 183]
BMP receptors	BMPRIB expression prefigures the cartilage primordia [184]	–	–	–
	BMPRIB present on chondrogenic cells, stimulation of BMPRIB expression by TGF-β [65, 185]	–	–	–
	Inhibition of BMPRIB expression by FGF causes apoptosis [80]	–	–	–
GDFs	Formation of joints and joint spaces [186, 187]	–	–	–

(*continued*)

Table 2 (continued)

Growth factor/ receptor	Role in the development of cartilage tissue in vivo	Role in adult cartilage tissue	Role in cartilage lesions or in articular cartilage disease	In vitro applications and functions
IGF family				
IGF-I	IGF promotes limb bud outgrowth [188–190]	IGF-I is detected in articular cartilage [115] and in synovial fluid [191]	Increased synthesis of IGF-I and IGFBP-3 in OA [192–194] and RA [194]	Chondrogenic effect on MSCs in the absence of insulin [195]
	Co-expression of IGF-I and IGFBP-2 in condensing mesenchyme [196]	Autocrine effect [197]	–	–
	Stimulation of IGF synthesis in growth plate by growth hormone [116]	–	–	–
	Expression mainly in proliferating chondrocytes of the growth plate [121]	–	–	–
IGF-II	Expression in chondrocyte precursors, co-expression with both types of IGF receptors [95]	Autocrine effect [197]	–	–
IGF binding proteins				
IGFBP-3	Perichondrium [94]	Transport of IGF-I	Inhibits of IGF-I induced matrix synthesis	Antiproliferative effect of IGFBP-3 on RCJ3.1C5.18 cell line [198]
	–	–	Increased in synovial fluid of OA and RA [194]	–
	–	–	Increased synthesis in OA [192, 193]	–
IGFBP-4	Developing cartilage [95]	–	–	–
IGFBP-5	Mesenchymal condensations [95]	–	–	–
IGFBP-6	Mesenchymal condensations [95]	–	–	–
IGF receptors	–	–	Increased number of binding sites in OA chondrocytes, decreased responsiveness to IGF [192]	–

PTHrP				
PTHrP	Synthesis in epiphyseal chondrocytes [67, 169]	–	Prevention of collagen type X synthesis by MSC of OA donors [144]	Prevention of collagen type X synthesis by bone marrow MSC [143, 144] and by adipose tissue derived MSC [143]
Hedgehog proteins				
Ihh	Production by prehypertrophic chondrocytes, induction of PTHrP in epiphyseal cartilage [68, 69]	–	–	–
Shh	Expression in the ZPA, role in AP axis formation [89, 199]	–	–	–
Growth factor interactions				
FGF/TGF	Stimulation of TGF synthesis by FGF in progress zone => promotes transition of precursor cells towards differentiation [46]	–	–	FGF suppresses senescence induced by TGF-β2 [129]
	–	–	–	"Secondary progenitor cells" from human articular chondrocytes [35]
	–	–	–	Stimulation of chondrocyte growth in vitro [203]
	–	–	–	Synergistic effect on chondrocyte proliferation together with PDGF-BB [124]
FGF/BMP	FGF and BMP have opposite effects on limb outgrowth [208]	–	–	–
	FGF and BMP interact to induce apoptosis of the interdigital mesenchyme [80]	–	–	–
IGF/TGF	–	–	–	Additive effect on chondrogenesis of MSC [138] and on synovium derived MSC [200]

(*continued*)

Table 2 (continued)

Growth factor/ receptor	Role in the development of cartilage tissue in vivo	Role in adult cartilage tissue	Role in cartilage lesions or in articular cartilage disease	In vitro applications and functions
	–	–	–	Enhanced chondrogenesis of periosteal explants [201]
	–	–	–	Synergistic effect of IGF-I and TGF-β on redifferentiation of human articular chondrocytes [202]
IGF/BMP	–	–	Synergistic effect on matrix synthesis in OA chondrocytes [204]	Synergistic effect on matrix synthesis [204, 206]
	–	–	Inhibitory effect on MMP-13 expression [205]	
IGF/TGF/FGF	–	–	–	Enhance proliferation of synovium derived stem cells [200]
IGF/BMP/FGF	–	–	–	Inhibition of anabolic effects of IGF-I and BMP-7 on chondrocytes by FGF [105]
TGF/BMP	–	–	–	Induction of chondrogenesis of adipose-derived stem cells by TGF-β3 combined with BMP-6 [135]
	–	–	–	Induction of chondrogenesis of bone marrow and adipose tissue derived MSC by TGF-β2 in combination with BMP-7 [136]
	–	–	–	Synergistic effect of TGF-β1 and BMP-2 on the chondrogenesis of bone marrow MSC [207]

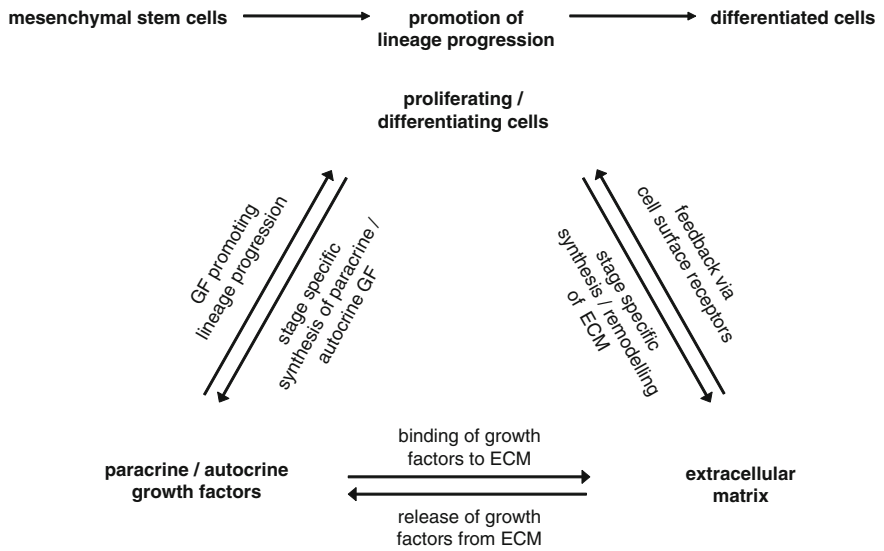

Fig. 1 Interaction of cellular differentiation, growth and differentiation factors, and specific matrix molecules during histogenesis. Morphogenesis and histogenesis result from the interaction of specific growth and differentiation factors acting in a stage dependent manner. Growth factor actions are modulated by binding to ECM molecules and the expression of their receptors depending on the developmental stage of the cells

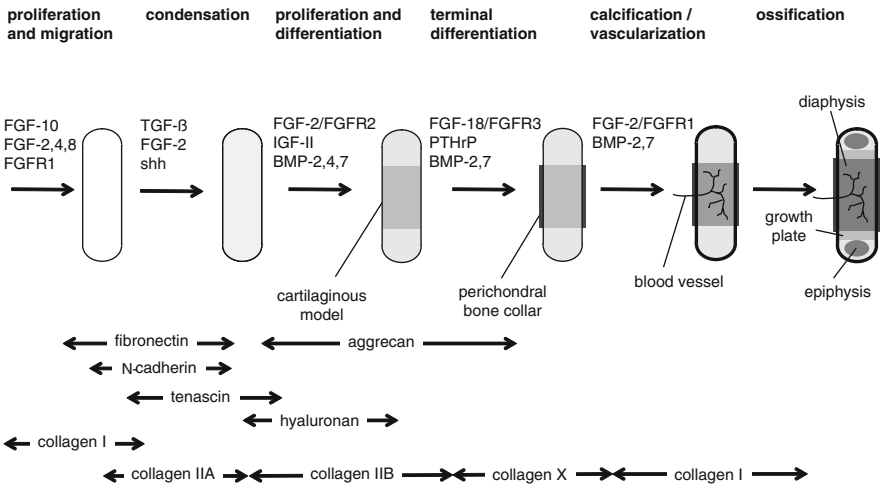

Fig. 2 Development of the long bones under the control of growth factors. The different developmental stages of the skeletal primordia are shown together with the specific growth and differentiation factors (*above the arrows*) which promote the stage specific synthesis of matrix molecules (*below*) [6, 144]

2.1 Migration and Proliferation

In the initial phase of limb development, mesenchymal cells of the lateral plate mesenchyme and of the somitic mesenchyme migrate towards the limb field [2]. The ectodermal cells are induced by the mesoderm to form a specialized epithelial structure termed the apical ectodermal ridge (AER). The AER supports migration and proliferation of the mesenchymal cells providing the cell mass for the formation of precartilage condensations [46, 47].

2.2 Cell Condensation

Cell condensation is characterized as a transient stage during the early morphogenesis which can be detected by PNA (peanut agglutinin) staining [48, 49]. During cell condensation, cell density in the prospective limb regions is increased leading to cell–cell contacts mediated by cell adhesion molecules such as N-cadherin and N-CAM [50–52] and the formation of gap junctions [53, 54]. Cell adhesion molecules are expressed specifically during the condensation phase and down regulated subsequently upon chondrogenic differentiation of the prechondrogenic cells. Prior to cell condensation, the extracellular matrix in the prospective limb regions contains high amounts of collagen type I and hyaluronan [55, 56]. Hyaluronan is supposed to prevent cell–cell interactions prior to the condensation phase [56]. During the condensation phase, hyaluronidase activity is detected, suggesting that matrix remodeling takes place allowing for cell–cell interaction. During the condensation phase, a specific splice variant of fibronectin, FnEIIIA, is detected throughout the condensations [57, 58]. Fibronectin was shown to be essential for condensation and subsequent chondrogenesis. Furthermore, fibronectin distribution during the condensation phase indicates the localization of skeletal elements formed later on [55].

2.3 Chondrogenic Differentiation

Cellular differentiation is characterized by the increased synthesis of transcription factors sox-5 and sox-6, and the appearance of the cartilage specific transcription factor sox-9 [59–61]. Collagen type I and fibronectin are synthesized in the ECM prior to condensation and reach a maximum density at the time of cellular differentiation [55]. Chondrogenic differentiation of the condensing cells is characterized by the appearance of collagen types II, IX, and XI, the characteristic components of collagenous network of cartilage tissue. As a result of chondrogenic differentiation,

remodeling of the extracellular matrix towards the cartilaginous composition of the tissue takes place. Collagen type I consequently disappears from the tissue [55]. In adult articular cartilage, fibronectin is detected predominantly in the pericellular area [62]. Collagen type I is usually not detected in articular cartilage unless repair tissue is formed originating from the bone marrow. Mixed collagen type I and type II formation resulting in mechanically inferior fibrocartilage is assumed to be an intrinsic property of MSCs which cannot be avoided in mesenchymal cartilage repair [63].

During cell differentiation, tenascin is transiently up-regulated in the developing tissue. Tenascin and its receptor syndecan prevent further interaction of N-CAM with fibronectin and thus terminate the condensation phase, allowing for further progression of cellular differentiation [64].

Along with cellular differentiation and cartilage tissue formation, the skeletal elements of the limbs are shaped into the precursors of the future skeleton. Joint formation and the organization of the digital rows involve apoptosis of the cell groups in the joint space and the interdigital regions. Therefore, the mesenchymal cells of the limb bud either undergo chondrogenic differentiation or apoptosis [65].

2.4 Endochondral Ossification

During the development of the appendicular skeleton in vivo, the cartilaginous models of the long bones are replaced by bone tissue. Thus, lineage progression towards terminal differentiation is an intrinsic program of the mesenchymal cells. Articular cartilage, however, does not undergo the process of terminal differentiation unless there are pathological conditions such as osteoarthritis (OA). In OA, lineage progression is resumed leading to the formation of collagen type X as a hypertrophy marker [66] and finally to abnormal calcification patterns of the cartilage matrix. The mechanisms preventing the chondrocytes of the periarticular region from terminal differentiation and maintaining the cartilaginous phenotype of articular cartilage are extensively studied in vivo [67–69]. In the growth plate, chondrocytes resume proliferation leading to columnar cartilage which represents the growth zone of the long bones in the embryo and the juvenile organism. Chondrocytes leaving the growth zone start synthesizing collagen type X which is the hallmark of hypertrophy. Morphologically, the chondrocytes swell and adopt the hypertrophic phenotype. The hypertrophic cartilage tissue is eroded by invading cells and replaced by bone marrow and in the end by bone. Hypertrophic chondrocytes also secrete matrix degrading enzymes such as MMP-13 which cleaves specifically collagen type II and thus contributes to remodeling of the extracellular matrix. Hypertrophic chondrocytes finally undergo apoptosis.

3 The Influence of Growth Factors on Cartilage Development In Vivo

An overview of the occurrence and actions of growth factors in developing and mature cartilage tissue is given in Table 2.

3.1 Fibroblast Growth Factors in Early Skeletal Development

The members of the FGF family share a homologous central core but differ in their carboxyterminal and N-terminal regions due to alternative splicing [70]. The FGFs are known as mitogens for mesodermal and neuroectodermal cell types. They induce chemotaxis and angiogenesis [71] and bind to heparan sulfate with high affinity [72]. Members of the FGF family of growth factors play a crucial role in the development and morphogenesis of the appendical skeleton. They are involved in all stages of tissue development as well as in the signal transduction during mechanical loading. FGFs are also involved in the remodeling and degradation of articular cartilage matrix taking place in inflammatory joint diseases.

FGFs are indispensable for outgrowth of limb buds preceding the development of the appendical skeleton [47, 73]. The initial steps involve epithelial to mesenchymal interactions mediated by members of the FGF-family. Mesenchymal FGF-10 and ectodermal FGF-8 are the earliest factors involved in limb bud outgrowth. FGF-10 is synthesized by the mesenchymal cells and induces the expression of FGF-8 within the AER. The proximo-distal axis of the developing limb is established via this feedback loop [74] which results in accumulation of proliferating cells in the region of the prospective limb bud [46]. A specific splice variant of CD44 with the ability to specifically bind FGF-8 mediates the presentation and thus the stimulation of mesenchymal cells by FGF-8 [75]. FGF-4 and FGF-2 are co-expressed in the AER [74, 76, 77]. Knowledge about these events arises from regeneration of limbs in amphibians [78] and knockout models in mice [74]. Signal transduction is mediated by FGFR2 [74].

During morphogenesis of the limb skeleton, the members of the FGF family of growth factors interact with other growth and differentiation factors. After the onset of condensation in the proximal region, a growth zone is formed between the AER and the condensing cell mass, termed the progress zone (PZ). The dynamic structure of the PZ results from the interaction of members of the FGF family with TGF-β. Proliferating cells move away from the AER and leave the zone governed by the growth promoting FGFs. Further proximally, members of the TGF-β superfamily are expressed within the condensing region of the limb bud. TGF-β promotes chondrogenic differentiation of the cells leading to the expression of cartilage matrix genes. Whereas TGF-β acts as growth stimulus in undifferentiated mesenchymal cells [79], it stimulates the formation of N-cadherin and N-CAM and

the synthesis of fibronectin and tenascin in the condensing regions, thereby promoting the progression of mesenchymal cells towards chondrogenic differentiation [50].

When limb outgrowth is completed, growth stimulation by the FGFs is terminated by regression of the AER induced by members of the BMP family [80, 81].

3.2 The TGF-β Superfamily of Growth and Differentiation Factors

The TGF-β superfamily of growth factors comprises a number of growth and differentiation factors characterized by their dimeric structure. Growth factors belonging to the TGF-β superfamily are the transforming growth factors TGF-β1, β2 and β3, the bone morphogenetic proteins (BMP-2–BMP-15), growth and differentiation factors (GDF), activin and inhibin, and Müllerian inhibitory substance. BMPs often act as heterodimers which are known to have higher affinities for their receptors [82]. Members of the TGF-β superfamily are involved in embryonic development as well as in repair responses to tissue injuries, but also in pathological tissue responses such as scarring and fibrosis [83].

Among the members of the TGF-β superfamily TGF-β1 to TGF-β3, BMP-2, -4, and -7, as well as GDF-5 and GDF-6, play crucial roles during skeletal development.

After the establishment of the AER, TGF-β1 is expressed in the condensing regions of the limb buds. It plays a crucial role in co-stimulating HA synthesis in the sub-epidermal layer of the limb bud [84]. TGF-β also induces the synthesis of cell adhesion molecules involved in prechondrogenic cell–cell interactions, N-CAM, and N-cadherin, which are prerequisite for the induction of chondrogenic differentiation [50, 85].

In the early phase of condensation, members of the TGF-β family stimulate the synthesis of fibronectin [86] which is necessary for the condensation and differentiation of the mesenchymal cells. Furthermore, members of the TGF-β family upregulate the synthesis of tenascin and collagen type I which is transiently expressed during the early phase of condensation [50]. TGF-β is known to induce the synthesis of the transcription factor sox-9 which is the key regulator of chondrogenesis [87]. The expression of Sox-9 is regulated by other paracrine factors such as FGF [88].

BMPs are co-expressed with FGFs in the AER but act as negative regulators of the AER [81] and as morphogenic factors involved in the antero-posterior axis formation induced by the zone of polarizing activity (ZPA) [89, 90]. BMPs originating from the AER are involved in shaping the autopod by inducing apoptosis in the interdigital mesenchyme acting through the BMP receptor BMPR1A which is expressed throughout the limb buds. In the digital elements, TGF-β1 induces the synthesis of BMPR1B which allows for chondrogenic differentiation of the

condensing cells mediated by BMP. BMPR1B expression is inhibited in the interdigital mesenchyme by FGFs secreted by the AER [80].

BMP-2, -4, and -7 were shown to be co-expressed throughout the limb bud. They are required during the condensation stage and later on for the induction of chondrogenic differentiation. Inhibition of BMP signaling by misexpression of noggin causes complete inhibition of prechondrogenic condensation [91]. After chondrogenic differentiation, BMP actions in the limb cartilages are regulated by their endogenous inhibitor noggin [91]. BMPs stimulate the synthesis of noggin and thus induces limitation of the chondrogenic regions in the limb. At the same time, BMP expression persists in the perichondrium and stimulates recruitment of mesenchymal cells for chondrogenic differentiation [91]. Thus, TGF and BMP pathways interact in the context of chondrogenic differentiation of mesenchymal cells.

3.3 The Role of IGFs in the Development of Cartilage Tissue

During embryogenesis, both isoforms of the IGF family, IGF-I and IGF-II, are present in the skeletal system. Absence of IGF signalling exerted by knockout of IGF receptor genes leads to severe skeletal defects [92]. Both receptors, IGFR1 and IGFR2, are expressed in all stages of limb bud development [93]. The IGF actions in the skeletal system are modulated by the IGF binding proteins (IGFBP) by reducing or enhancing the bioavailability of the IGFs. IGF-I and IGF-II and the IGFBP exhibit a specific pattern in the developing limb buds, suggesting a specific role during chondrogenesis [94]. In prechondrogenic condensations, IGF-II is found to be co-expressed with IGFBP-5 and IGFBP-6 [95]. During digit formation, IGFs are also present in the interdigital mesenchyme undergoing apoptosis instead of chondrogenic differentiation. IGFBP-2, -4, and -5 are found in the interdigital zone whereas IGFBP-3, -4, and -5 are detected in the phalangeal joint areas [94] at the time of joint formation. Components of the IGF-system are also detected in the AER (IGFBP-2 and -4) and in the ZPA (IGF-I and IGFBP-4) [94].

3.4 Terminal Differentiation or Development of Articular Cartilage

During further progression of skeletal development, two divergent lineages of chondrocytic phenotypes emerge from the cartilaginous models of the skeleton. Periarticular cartilage is prevented from terminal differentiation and develops into hyaline articular cartilage. Cartilaginous tissue of the growth plate on the other hand progresses towards terminal differentiation and is finally replaced by bone. The regulation of terminal differentiation vs maintenance of epiphyseal cartilage has been extensively studied in vivo. Vortkamp et al. have established a model of

interdependent regulation by a negative feedback loop between the growth zone and the epiphysis [69]. Prehypertrophic chondrocytes synthesize Ihh, which induces PTHrP in epiphyseal chondrocytes. It is assumed that PTHrP is a key factor preventing hypertrophy and terminal differentiation of the epiphyseal chondrocytes. TGF-β2 was shown to stimulate the synthesis of PTHrP in the perichondrium of organ cultures of developing bones. TGF-β expression was induced by Hedgehog proteins [96]. BMP acts independently of PTHrP on terminal differentiation by delaying this process [68].

4 Growth Factors in Adult Cartilage Tissue

Growth and differentiation factors are also present in adult articular cartilage performing distinct functions in tissue maintenance, transduction of mechanical stimuli, and regeneration. Since these functions differ from the actions exerted during development, the availability of growth factors is adjusted by specific binding factors and antagonists, and by their affinity to extracellular matrix molecules such as heparan sulfate, collagen, and fibronectin. These mechanisms make sure that the growth factors are available to regulate the natural turnover of cartilage matrix or induce tissue repair in cartilage injury for example caused by traumatic tissue damage and disease.

4.1 The Role of FGF in Adult Articular Cartilage

Besides their role in the development of the skeleton, the FGFs are also detected in hyaline articular cartilage. FGF-2 was found to be entrapped within the pericellular matrix bound to heparan sulfate side chains of perlecan [97]. Perlecan was detected in the pericellular matrix of the chondrocytes rich in collagen type VI. FGF-2 has been demonstrated to exert significant functions during mechanical loading of articular cartilage [98] and mediate the immediate response of articular cartilage to mechanical injury [99]. Its role in cartilage injury and repair is still controversial, since FGF-2 is known to have anabolic as well as catabolic effects. It was shown that FGF-2 inhibited aggrecanolysis by ADAMTS, which is regarded as an initial and still reversible step of matrix degradation in OA and rheumatoid arthritis (RA) [100]. On the other hand, FGF-2 is known to induce matrix metalloproteinases MMP-1 and MMP-3 and tissue inhibitor of metalloproteinase TIMP-1 [99]. FGF-2 stimulates the synthesis of MMP-13 [101], the major collagen type II degrading enzyme which is found to be present in excess in OA and RA, but also plays a role in tissue remodeling during skeletogenesis [102, 103]. Furthermore, FGF-2 induces chondrocyte proliferation and thus might contribute to cluster formation in affected cartilage tissue. Nucleus pulposus cells up-regulate the synthesis of noggin upon stimulation with FGF-2, which might contribute to the reduced sensitivity to

BMP-7 observed in diseased tissue [104, 105]. Increased synthesis of FGF-2 by cells of the synovial tissue contributes to the altered growth factor environment in cartilage disease [103]. The multi-facetted actions of FGF-2 in healthy hyaline cartilage and in trauma and disease demonstrate the imperative of tight regulation of the availability of growth factors in the adult cartilage tissue.

4.2 Members of the TGF-β Superfamily in Adult Articular Cartilage

TGF-β is secreted together with its propeptide (latency associated peptide, LAP), which binds with high affinity to TGF-β and thus limits its bioavailability. Furthermore, there are several members of LTBP proteins which bind to TGF-β and form large latent complexes (LLCs). LTBPs contain binding domains for extracellular matrix proteins such as collagen and fibronectin. LTBP-3 knockout mice show skeletal abnormities which are due to impaired TGF-β signaling leading to early OA [106]. Thus LTBP-3 is assumed to have a regulatory function in articular cartilage. LTBP-1 plays a major role in the growth plate during endochondral ossification [107, 108]. These observations indicate that local concentrations of TGF-β are tightly regulated by binding factors and that sequestered growth factors might be released upon demand [109]. TGF-β itself is known to be essential for the homeostasis of adult articular cartilage, because the phenotype of TGF-β receptor type II knockout mice displays signs of OA and impaired cartilage repair [110]. On the other hand, overexpression TGF-β in mice leads to the formation of osteophytes similar to osteophyte formation in OA [111]. Inflammatory cytokines such as IL-1 induce the synthesis of TGF-β3 in articular chondrocytes [112].

The role of BMPs is largely investigated during development of the skeleton and well established in cell differentiation, histogenesis, and morphogenesis. BMPs are also known to be present in synovial fluid and mature articular cartilage tissue [113]. BMP signaling is known to be strictly regulated by negative feedback, for example by noggin, which is induced by various isoforms of BMP. BMP overexpression leads to progression of differentiation and to matrix calcification, even in articular cartilage. Therefore it can be hypothesized that there are mechanisms to limit BMP signaling in the adult articular cartilage tissue. TGF-βs are known to inhibit partially excess BMP action, but the interactions of TGF-β and BMP during development and in mature articular cartilage tissue have not been fully elucidated.

4.3 The Role of IGF in Adult Articular Cartilage

IGF was first described to stimulate proteoglycan synthesis [114] and to maintain a steady state of proteoglycan synthesis in articular cartilage explants [115]. In the

growth plate, IGF was shown to be the local mediator of growth hormone action on proliferative chondrocytes and thus to contribute to the longitudinal growth of the long bones [116, 117]. The IGFs are found as complexes with their specific binding proteins, themselves exerting distinct functions promoting or inhibiting IGF signaling. Endocrine IGF-I in the bloodstream is complexed by IGFBP-3 as a carrier protein. In articular cartilage, IGFBP-3, -4, and -5 were detected [118]. IGFBPs are known to play significant roles in IGF transport in the articular cartilage [119] and in transduction of mechanical stimulation [120]. IGFBPs bound to extracellular matrix might represent a reservoir for IGF within the cartilage tissue. IGFBP-3 was shown to bind to fibronectin of the pericellular matrix, but neither IGFBP-4 nor IGFBP-5 were associated with these extracellular matrix components [118]. On the other hand, in OA, IGF-signaling and thus matrix synthesis are impaired by the increased levels of inhibitory IGFBP-3 in the synovial fluid [121]. Although IGF-I is synthesized in the diseased articular cartilage at higher rates compared to healthy cartilage tissue, the stimulatory effect on matrix synthesis is reduced compared to normal articular cartilage tissue [122].

5 Engineering of Cartilage Tissue In Vitro

An overview of the applications and actions of growth factors in cartilage cultivation in vitro is given in Table 2.

5.1 Expansion of Progenitor Cells

For the expansion of chondrocytes or MSCs, various growth factors have been used in order to optimize cell growth starting with a limited number of primary cells. The expansion of chondrocytes usually implies a process of dedifferentiation, meaning that matrix synthesis is down-regulated when chondrocytes are isolated from their natural environment. Growth factor treatment stimulates the growth of chondrocytes in vitro, but also contributes to dedifferentiation [33].

Among the members of the FGF family, FGF-2 is the factor most studied in cartilage tissue engineering. Treatment with FGF-2, which is a strong mitogen for various mesenchymal cell types, leads to a rapid loss of collagen type II synthesis during cell expansion. On the other hand, FGF-2 inhibits the formation of stress fibers and the typical collagen type I expression of dedifferentiated articular chondrocytes [123]. Furthermore, chondrocytes expanded in the presence of FGF-2 are able to produce higher amounts of matrix constituents when culture conditions permissive for redifferentiation are applied [123]. Various growth factor regimes have been suggested for the expansion of articular chondrocytes, most of them comprising the application of FGF-2, which allows for the expansion of adult articular chondrocytes and at the same time priming the cells for

optimized redifferentiation. FGF-2 is also used in combination with TGF-β [35] or TGF-β and PDGF-BB [124].

FGF-2 together with TGF-β and PDGF-BB was also shown to promote dedifferentiation towards the "mesenchymal" phenotype, thus leading to increased synthesis of cartilage matrix components upon the application of differentiating conditions [33]. This beneficial effect on chondrocytes is contributed to the maintenance of sox-9 expression during expansion of articular chondrocytes and MSCs in vitro [88].

For mesenchymal progenitor cells, FGF-2 is a very powerful growth stimulating factor, extending the life span of bone marrow MSC [125], but also helping to maintain the multipotential properties of the precursor cells during prolonged cultivation in vitro. This has been shown for the osteogenic [126] and for the chondrogenic potential of bone marrow MSC [127]. The mechanism by which treatment with FGF maintains the differentiation potential of MSCs and improves the matrix synthesis of dedifferentiated chondrocytes is not yet clear. Varas et al. have shown that treatment with FGF-2 during extended expansion of MSCs leads to up-regulation of the cartilage specific integrin alpha10 while reducing the expression of integrin alpha 11 characteristic for fibroblasts [128]. FGF-2 was also shown to slow down senescence of MSCs during expansion in vitro by inhibiting TGF-β2 expression [129].

In chondrogenic cultures of MSCs, FGF receptors are expressed in a similar manner as in vivo during chondrogenesis. FGFR1 was detected in MSCs prior to chondrogenic differentiation and later on in hypertrophic constructs derived from pellet cultures of human MSCs [130, 131]. On the mRNA level, FGFR2 and FGFR3 expression was shown to increase over time after the onset of chondrogenic differentiation [131], whereas on the protein level, FGFR2 and FGFR3 were not detected in collagen type II synthesizing tissue or in articular cartilage at all. The three types of FGF receptors were detected on the protein level in pellet cultures of adult human MSCs, indicating a role for FGF during the late phase of cartilage differentiation in vitro [130]. Hypertrophy and calcification were accompanied by expression of MMP-13 and decreasing levels of proteoglycan and collagen content, indicating that matrix degradation takes place in a way comparable to the in vivo situation in the growth plate [131].

5.2 Chondrogenic Differentiation In Vitro

Chicken limb bud mesenchymal cells, long established as in vitro model of chondrogenic differentiation, demonstrate the ability of mesenchymal cells to undergo reversibly differentiation, terminal differentiation, and dedifferentiation [28]. Suspension cultures of these mesenchymal cells undergo spontaneous chondrogenesis and start collagen type II synthesis in vitro induced by cell–cell contacts upon aggregation. The differentiating cells in aggregate cultures were shown to proceed towards terminal differentiation and synthesize collagen type

X [132]. On the other hand, chicken limb bud cells adopt a fibroblastic morphology and synthesize collagen type I when transferred to monolayer culture at low cell densities.

For chondrogenic induction of adult MSCs, high cell densities in pellet culture or in appropriate 3D biomaterials in combination with growth factors are required. Growth factors currently used for chondrogenic differentiation of MSCs belong to the TGF-β superfamily. TGF-β and BMPs are well established in redifferentiation of culture expanded chondrocytes [33, 123, 124]. The protocols for chondrogenic differentiation of bone marrow MSCs make use of TGF-β1 [133], TGF-β3 [10, 36] BMP-2, -4, -6, and -7, or combinations of these growth factors. Most of these protocols use serum free conditions and include dexamethasone and ITS (insulin, transferrin, and selenite) supplement in the culture medium [10]. Since MSCs can be isolated from different tissues, the growth factors used for chondrogenic induction are adapted to the different requirements. For the chondrogenic differentiation of bone marrow MSCs, BMP-2 was most effective in comparison with BMP-4 and BMP-6 [134].

Compared to bone marrow MSCs, adipose tissue-derived MSCs have a reduced chondrogenic potential. In this cell culture system, the combined action of TGF-β and BMP-6 was most effective in inducing chondrogenesis. This was due to upregulation of the TGF-β receptor-1 (TβRI) by BMP-6. TβRI is usually absent from a adipose tissue-derived MSCs [135]. A combination of TGF-β2 and BMP-7 also effectively induced chondrogenesis of adipose tissue-derived MSCs [136].

Bovine synovium derived MSCs cultivated in alginate gel underwent chondrogenic differentiation upon treatment with BMP-2 but not in response to TGF-β [15]. Micromass cultures of synovium derived cells and explants of the synovial membrane were differentiated towards the chondrogenic lineage using TGF-β, BMP-2, or BMP-7, in the absence of dexamethasone. These growth factors induced different types of cartilaginous tissue, all showing synthesis of cartilage matrix proteoglycans. Collagen type II was only obtained by treatment with BMPs, but not with TGF-β [137]. Morphologically, BMP driven chondrogenesis resulted in cartilage-like appearance of chondrocytes within lacunae, but similar to hypertrophic cartilage, which was confirmed by expression analysis of collagen type X. On the other hand, TGF-β1 failed to induce collagen type II synthesis and showed lower levels of collagen type X. Morphologically, lacunae within the tissue were not apparent.

Members of the IGF family have also been used in protocols for chondrogenic induction of MSCs. Martin et al. have shown that IGF-I and -II stimulate the growth of marrow derived cells in vitro, but have no influence on subsequent chondrogenic or osteogenic differentiation [126]. In vitro induction of chondrogenesis usually is carried out under serum free conditions using a pre-mix of ITS (insulin, transferrin, selenite). Longobardi et al. pointed out [138] that high concentrations of insulin in the culture medium might obscure the effect of IGF-I on chondrogenic differentiation of MSCs since insulin also binds to IGF receptors, although with much lower affinity. In the absence of insulin, IGF-I stimulated cell proliferation as well as chondrogenesis of MSCs and induced Sox-9 and collagen type II expression and

proteoglycan synthesis in a way that was comparable to TGF-β. Effects of IGF-I and TGF-β were additive. In contrast to IGF-I, TGF-β induced condensation as shown by PNA staining and the expression of cell adhesion molecules mediating cell–cell contacts such as N-cadherin. Although IGF-I induced the synthesis of chondrogenic markers, this was carried out in a way that was independent of cell condensation [138].

5.3 Maintenance of the Hyaline Phenotype in Cultivated Cartilage Tissue

For future clinical applications of MSCs for the treatment of articular cartilage defects, the characterization of cellular differentiation and the composition of the resulting cartilage tissue are essential in order to make sure that implanted tissue will meet the biochemical and biomechanical requirements. To date there are major problems related to the characterization of the developmental stage of the resulting tissue. In chondrogenic cultures of MSCs, collagen type I is usually expressed along with collagen type II indicating a fibrocartilaginous phenotype of the cells in vitro [139, 140]. Fibrocartilage is biomechanically inferior to hyaline cartilage and thus the implantation of fibrocartilaginous tissue might lead to failure of the graft [63].

Another concern is the synthesis of collagen type X and the progression towards terminal differentiation observed in cartilaginous tissue derived from various sources of MSCs. Terminal differentiation might lead to calcification and invasion of blood vessels and thus to the loss of the implanted tissue [45, 139, 140]. Early induction of collagen type X was observed upon chondrogenic induction of MSCs, even before the onset of collagen type II synthesis [141, 142]. In order to overcome this problem, PTHrP was applied in chondrogenic cultures to prevent terminal differentiation. In the presence of PTHrP, collagen type X expression was suppressed and alkaline phosphatase activity was reduced in comparison with control cultures [143] even when derived from OA patients [144].

6 Conclusion

The development of the limbs is one of the most studied models for the investigation of the mechanisms controlling morphogenesis and regeneration in vivo. In vitro culture models have been derived from cell cultures of the limb mesenchyme to explore the developmental mechanisms under defined conditions and knockout mice have been generated to study in detail the factors contributing to tissue development and morphogenesis.

In vitro synthesis of cartilage tissue, mainly driven by the emerging concepts of regenerative medicine, relies on experimental approaches involving biomaterials

and growth factors known to support chondrogenesis in vivo. Besides the actions of growth factors, there are significant influences of the ECM components on cartilage differentiation which might have a major impact on the design of new biomimetic scaffolds. These effects mediated by the cooperation of growth factors and their specific receptors with integrins and other cell surface receptors were shown to play a major role in modulating growth factor actions during differentiation.

Regarding future clinical applications of cartilage engineering from MSCs, there are still important questions to be answered regarding the differences between permanent articular cartilage and the transient cartilage of the growth plate. Knowledge about the mechanisms defining the type of cartilage formed in vivo might lead to improved protocols for cartilage production in vitro.

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Outgrowth Endothelial Cells: Sources, Characteristics and Potential Applications in Tissue Engineering and Regenerative Medicine

Sabine Fuchs, Eva Dohle, Marlen Kolbe, and Charles James Kirkpatrick

Abstract Endothelial progenitor cells from peripheral blood or cord blood are attracting increasing interest as a potential cell source for cellular therapies aiming to enhance the neovascularization of tissue engineered constructs or ischemic tissues. The present review focus on a specific population contained in endothelial progenitor cell cultures designated as outgrowth endothelial cells (OEC) or endothelial colony forming cells from peripheral blood or cord blood. Special attention will be paid to what is currently known in terms of the origin and the cell biological or functional characteristics of OEC. Furthermore, we will discuss current concepts, how OEC might be integrated in complex tissue engineered constructs based on biomaterial or co-cultures, with special emphasis on their potential application in bone tissue engineering and related vascularization strategies.

Keywords Bone tissue engineering, Co-culture models, Endothelial progenitor cells, Vascularization

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1 Introduction

The creation of adequate tissue equivalents and other therapeutical products in tissue engineering and regenerative medicine is, for several reasons, a highly challenging task. First of all, tissues reveal a highly complex structure, usually consisting of more than a single cell type and a cellular organization in an individual, tissue specific manner. In addition, tissue function is regulated by a close interaction of the individual cell types controlled via matrix components and cell to cell communication mechanisms also underlying the control through physiological conditions such as oxygen tension, state of inflammation, or mechanical stimulation. Last but not least, tissues are integrated into the body and linked to central body functions, such as vascularization, ensuring the supply with oxygen and nutrients, as well as the removal of waste products. Furthermore, adequate vascularization is an essential prerequisite allowing stem cells to approach the sites of tissue repair [1–4]. The development of new therapies leading to a fast and successful vascularization is therefore one of the most central and highly discussed subjects in tissue engineering and regenerative medicine. In this context, the use of endothelial progenitor cells for proangiogenic cell therapies has been proposed as a potential means to overcome the current problems in the neovascularization of bioengineered or harmed tissues [4, 5]. Despite high expectations, the use of endothelial progenitor cells is currently not yet feasible for broad clinical applications, due to a series of open questions regarding the definition of the relevant cell types and the transfer into practicable approaches, which meet the clinical requirements.

2 Endothelial Progenitor Cells in the Neovascularization Process

For many years angiogenesis, the generation of blood vessels from the existing vasculature through activation of proliferation and sprouting mechanisms in adult endothelial cells, was considered as the exclusive pathway for blood vessel formation in an adult organism.

The understanding of blood vessel formation has changed in recent years, mainly due to the discovery of so-called endothelial progenitor cells by Asahara et al. [6]. They postulated that in the adult organism endothelial progenitor cells contribute to de novo formation of blood vessels in a process described as vasculogenesis. Since then, vasculogenesis, the contribution of stem cells or endothelial progenitor cells to de novo vascularization, has been discussed as a collateral mechanism in neovascularization. Although the definition clearly differentiates the two processes in the academic sense, both mechanisms seem to work hand in hand in blood vessel formation and remodeling. Both pathways are coupled through a series of mostly unknown signaling mechanisms guiding endothelial progenitor cells to the sites of repair and inducing their differentiation and functional integration into the vasculature [7].

A significant number of studies on endothelial progenitor cells have been published over the last decade, defining endothelial progenitor cells by surface markers such as CD133 and CD34 [8–10], and a series of other characteristics such as low density lipoprotein (LDL)-uptake [4] and binding of ulex europaeus agglutinin (UEA) [6, 11]. The ability to form vascular structures in proangiogenic matrices in vitro, as well as the potential to contribute to the vascularization in vivo have been defined as functional key elements of endothelial progenitor cells. Several sources, isolation procedures, and suggested marker profiles for endothelial progenitor cells have been described, although there is still a lack of consensus on which cell type would resemble the "true endothelial progenitor cell" or might be preferred in terms of a therapeutical application. From a practical point of view, such a cell would have to fulfill a series of prerequisites including: (1) origin from an easy obtainable source in connection with a minimal invasive procedure and (2) a good expansion capability not interfering with the therapeutical potential. According to these requirements, endothelial progenitor cells, in particular from peripheral blood or cord blood, have raised significant interest.

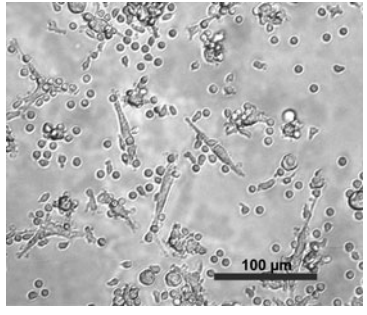
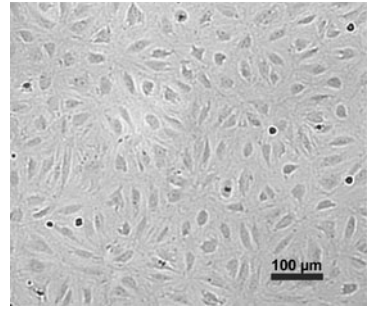
3 Diversity of Endothelial Progenitor Cell Populations

Experimental evidence from studies by Lin [12], Gulati [13], Hur [14], and Ingram [15] suggested that endothelial progenitor cells from the peripheral blood are a heterogeneous population of cells. Endothelial progenitor cells from peripheral blood were classified by several groups according to their order of appearance and morphological characteristics in early or late endothelial progenitor cells [14]. Other synonyms for late endothelial progenitor cells include outgrowth endothelial cells (OEC) [12], late OEC, blood OEC, and endothelial colony forming endothelial cells. Although the isolation procedure or the nomination of these subsets differs slightly amongst the individual studies, the phenotypic and functional key features described for those cells are more or less identical and are summarized in Table 1. This rather simple classification was at least a basis for the definition of blood derived endothelial progenitor cells for subsequent studies using these cells for applications in tissue engineering and regenerative medicine as described in this review.

The isolation of mononuclear cells from the blood by ficoll gradient centrifugation followed by culture in a commercial available cell culture medium resulted in cellular colonies with cobble stone-like morphology showing remarkable similarities to endothelial cells in terms of the marker profile and functional key elements (Table 1).

Those cells appear at relatively low frequency in the peripheral blood [15, 27, 28]. In contrast to these cells, so-called early endothelial progenitor cells make up the majority of mononuclear cells derived from the peripheral blood and show a more or less rounded to fibroblast-like morphology. Although they express to some

Table 1 Overview of characteristics and functions

	EPC	OEC
Morphology	Spindle shaped morphology [12, 16, 17]	Cobblestone-like morphology [13, 15, 18, 19]
		
Order of appearance	After 7 days in culture [14, 16]	After 2–3 weeks in culture [12–14]
Marker profile	CD31+ [6, 11, 17]	CD31+ [13, 19, 24]
	CD45+ [17, 20]	CD45− [20]
	CD34+ [6, 11, 16]	CD34+ [8, 16, 20]
	CD14+ [13, 16, 17]	CD14− [13, 16]
	CD146− [21]	CD146+ [21, 24]
	CD133+ [8, 9, 22]	CD133− [20]
	Flt-1 [14]	Flt-1 [14]
	eNos[14]	eNos [13, 14]
	vWF [14, 19, 23]	vWF [14, 19, 24]
	VE-Cadherin [11, 14, 17]	VE-Cadherin [12, 14, 24]
	KDR [11, 14, 23]	KDR [14, 16, 20]
	CD36+ [12]	CD36+ [12]
	Tie2+ [6, 13]	Tie-2+ [13]
		Caveolin-1 [13, 24]
Characteristics		
In vitro	Low proliferative potential [14, 16, 17]	High proliferative potential [14, 16]
	No tube formation on matrigel [16, 25]	Tube formation on matrigel [16, 25]
In vivo	Vasculogenic potential through paracrine mechanisms [13]	High vasculogenic potential [13, 14, 26]
	Secretion of proangiogenic molecules [16]	

extent relevant endothelial markers such as CD31 (compare Table 1), these cells do not show the full marker profile of endothelial markers or the functional features typical for endothelial cells. Most of these cells still carry hematopoietic markers such as CD45 (compare Table 1). Different studies have compared the early vs late endothelial progenitor cells in terms of their characteristics. The isolation of OEC has been reported from different sources such as human cord blood as well as adult peripheral blood or from different species such as porcine [29], murine [30], and canine [31].

Surprisingly, although only OEC showed the characteristics and angiogenic potential of endothelial cells in vitro, both cell types contributed to the de novo vascularization in vivo [14]. In a following study the same group provided additional data potentially explaining the discrepancies in the angiogenic potential of early EPC in vitro and in vivo. In this study early EPC supported the angiogenic activity of OEC in vitro [16] probably through paracrine mechanisms based on the production of IL-8 and VEGF by early EPC. This cytokine production improved the angiogenic activity of OEC in vitro. In addition, the invasion of OEC into angiogenic matrices in vitro was positively influenced when both cell types were applied mediated through activation of matrix metalloproteinases such as MMP-2 in OEC and MMP-9 in early EPC. In the same study a synergistic effect was also documented for the neovascularization process in vivo by the application of both populations in a hindlimb model. Subsequently, data from these studies are questioning the contribution of early endothelial progenitor cells to the neovascularization process as a result of a true progenitor function in a strict sense including the differentiation towards mature endothelial cells. It seems to be more adequate to assume that only a small subset contained within this mixed population constitutes a true endothelial stem progenitor cell with the ability to differentiate towards an endothelial cell. On the other hand, the improvement of the vascularization process by so-called early endothelial progenitor cells seems to be mainly the result of other mechanisms such as the support of mature endothelial cells or true stem cells by paracrine factors and other mechanisms which still need to be defined further.

4 Origin of OEC and Enrichment Strategies

Although it has been widely accepted that peripheral blood contains stem cells with the potential to differentiate towards cells with endothelial phenotype there are still a series of difficulties identifying their distinct origin. Several markers have been suggested in attempts to define the origin of OEC or to isolate them in a more specific manner from diverse mononuclear cell fractions. Nevertheless, most of these surface markers result only in an enrichment of cell populations containing OEC. In addition, some of these applied surface markers are not suitable to distinguish progenitor derived endothelial cells from mature endothelial cells circulating in the blood stream, as discussed in the following section.

Gulati et al. [13] isolated and identified OEC from the CD 14 negative fraction of mononuclear cells from the peripheral blood. Other approaches used CD146 to isolate cells with the characteristics of OEC from the cord blood and adult peripheral blood [12, 21]. The adhesion molecule CD146 is expressed in different types of endothelial cells throughout the vascularization and is often used to identify circulating mature endothelial cells (CEC) [32]. Delorme et al. used a combination of an adhesion step in order to separate mature endothelial cells from the mononuclear fraction followed by a magnetic separation step for CD146 positive cells. Using this approach they identified two distinct populations in cord blood and in peripheral blood forming OEC in culture CD146 positive EPC (CD146+, CD34+, CD45+, CD133+, or CD117+) and CD146 CEC (CD146+, CD34+, CD45−, CD133−, or CD117−).

The complexity of the problem of how to differentiate between endothelial cells from the circulation and from cells which have been mobilized from the bone marrow was highlighted by a study of Lin et al. [12]. This group investigated OEC from recipients of gender-mismatched bone marrow transplantations. By in situ hybridization, Lin et al. were able to identify endothelial cells from the recipient making up the majority of endothelial cells in the early phases of the culture. On the other hand, the donor derived endothelial cells constitute the major population after 1 month of the cultures which was due to their high expansion capacity (over 1,000-fold over 2 months). It was not possible to distinguish donor or recipient derived OEC on the basis of a marker profile, whereas the capability of expansion was one of the remarkable differences of the two endothelial cell populations found in the peripheral blood. Both endothelial cell types were positive for CD36, indicating their microvascular endothelial phenotype. This microvascular phenotype of OEC was also supported by findings from other groups. Microvascular characteristics of OEC were recently also supported by microarray data, indicating that OEC are different from macrovascular cells, share similarities with microvascular cells or might be even considered as an individual subclass of endothelial cells [33].

Although CD133 is one of the markers suggested for endothelial progenitor groups, cell sorting experiments provided evidence that OEC are not derived from CD133 or CD45 positive cells but seem to be enriched in the CD34 positive, CD45 negative fraction [20].

Besides these surface marker based isolation strategies, another approach to enrich OEC from the mononuclear fraction has been described recently [28]. By including a passaging step (Fig. 1a) in the early phase of the culture of mononuclear cells, the formation of OEC colonies was significantly improved in one group of cultures classified as high colony forming cultures. This simple protocol modification resulted in a significant enrichment of OEC (Fig. 1b) and in a higher number of OEC gained per individual donor. Although the reason for this effect has to be further defined, the protocol modification might exert a positive effect, specifically on such OEC with a high clonogenic potential, which have been described by several groups as stated above.

Other groups modified the isolation protocols by using full blood preparations omitting widely used centrifugation steps which resulted in an improved OEC

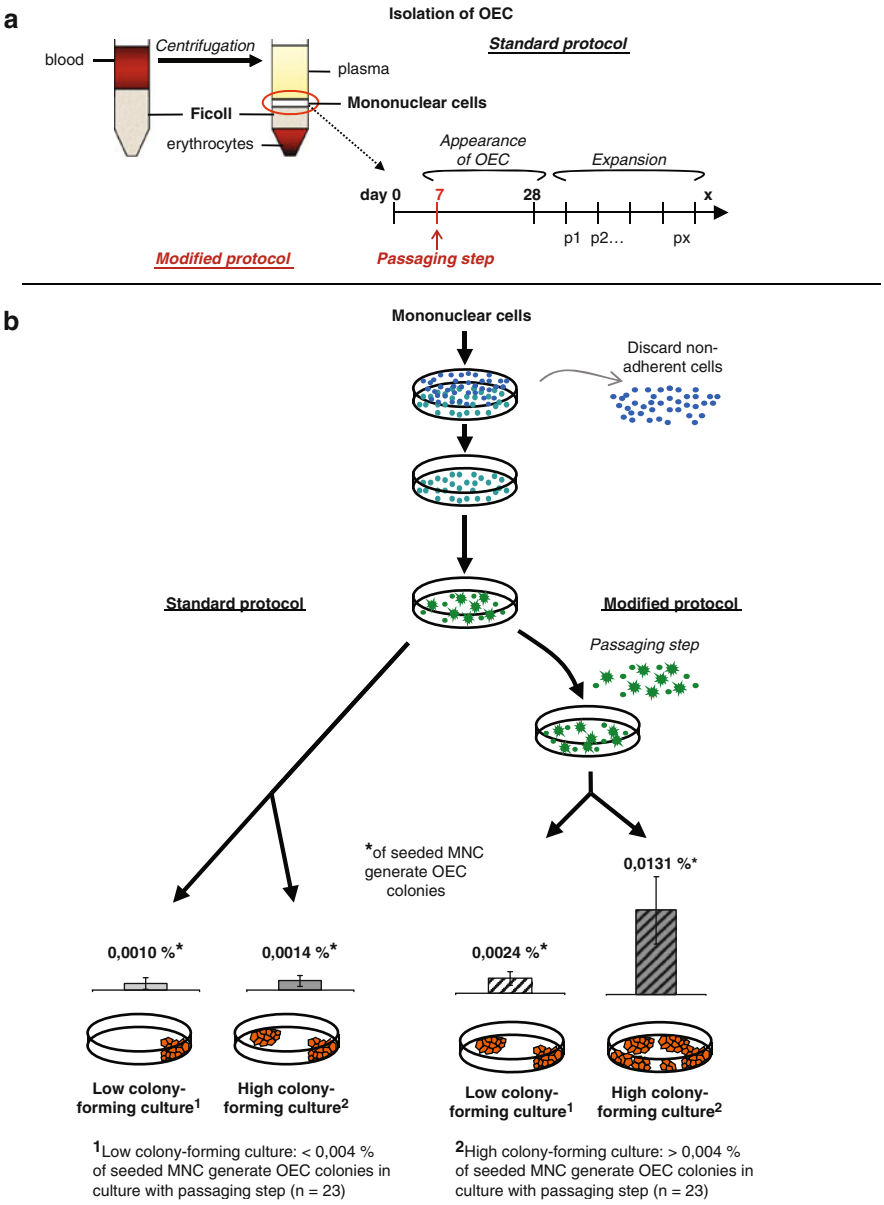


Fig. 1 (**a**) Schematic overview of the standard and the modified culture protocol according to Kolbe et al. (**b**) Schematic overview on the effects of protocol modification on the enrichment of OEC from adult peripheral blood

colony formation. For the further expansion of these cells the same group established culture conditions designed for a potential therapeutical use of OEC in humans [34].

Nevertheless, despite the advances in the understanding of endothelial progenitor cell biology, the identification and specific isolation of OEC from heterogeneous peripheral blood cell populations remains also a technical problem due to their very low frequencies in heterogeneous cell populations isolated from the peripheral blood. Steurer et al. [35] recently compared real time PCR and flow cytometry to detect circulating endothelial cells and endothelial progenitor cells. Using defined numbers of circulating endothelial cells added to heterogeneous blood mononuclear cells, detection limits were in the range of 0.001% for quantitative real time PCR or 0.01% for flow cytometry. Despite the ten-times higher sensitivity of real time PCR, the specificity of the real time PCR was lower due to the methodological problems associated with this technology also due to the lack of markers exclusively expressed on the relevant cell types.

5 In Vivo Evaluations of Angiogenic Potential of OEC

A series of studies investigated the in vivo potential of OEC for vascularization strategies in tissue engineering and regenerative medicine. From these studies we have learned that the successful formation of blood vessels depends strongly on the experimental settings and the source of OEC as described in the following section. Melero-Martin et al. [36] co-implanted OEC from the cord blood or adult peripheral blood together with smooth muscle cells in matrigel plugs. Only in co-implantion approaches blood vessel formation by OEC was observed, reflecting an active role of the smooth muscle cells in the blood vessel formation or stabilization. The angiogenic activity of the blood derived cells reduced with increasing numbers of passages that the cells underwent during their expansion in vitro. Consistent with the finding that the therapeutical potential correlates with cellular aging, cord blood derived endothelial cells had a higher angiogenic potential then those derived from adult blood. This observation was also confirmed by Au et al. [37] although this group used different experimental settings and co-implanted the endothelial cells with 10T1/2 mouse embryonic fibroblasts as stabilizing perivascular-like cells in collagen gels. In this study, co-implantation with other cell types such as 10T1/2 had no effect on the blood vessel formation itself. Nevertheless, the stability of newly formed blood vessels formed by adult peripheral blood derived cells was relatively impaired, so that these vessels regressed within the 3 weeks. Compared to this cord blood cell derived vessels revealed a stability lasting over months. In another study by Au et al. the critical role of stabilizing cells for the in vivo outcome of transplanted endothelial cells was proven in another experimental set up based on HUVEC as endothelial cell source and on bone marrow mesenchymal stem cells functioning as cells with perivascular potential. Using collagen gels based approaches, the blood vessel formation without mesenchymal cells (MSCs) was negligible whereas the addition of those or of 10T1/2 fibroblasts leaded to blood vessels with a long-term stability [38].

6 OEC for Vascularization of Complex Tissue Engineered Constructs

Successful regeneration or replacement of complex tissues combines challenges in biomaterial design, evaluation of therapeutically relevant cell populations, as well as an improved knowledge of cellular and molecular mechanisms involved in tissue repair. A fast and successful vascularization of such complex constructs is essential for the tissue survival but still associated with severe problems especially in term of larger or highly vascularized tissues such as bone or muscle. To overcome these limitations in tissue engineering, different strategies have been developed, including diverse delivery systems for angiogenic growth factors [39–41], as well as the generation of prevascularized tissues by incorporation of endothelial cells [42–45] or endothelial progenitor cells, recently also reviewed by [46]. Prevascularization and anastomosis of bioengineered vascular structures with those in the peri-implant tissue might be a suitable therapeutic approach to enhance or to accelerate the vascularization as recently shown by Levenberg et al. for a muscle construct [47].

Although OEC seem to possess a promising angiogenic potential, the question, how OEC could be applied for complex and properly vascularized tissue constructs, has to be addressed from case to case depending on the field of application. OEC have been used to generate vascular network in skin substitutes based on decellularized dermis [48, 49] leading to the active perfusion of the skin constructs by OEC derived blood vessels. Another potential application of outgrowth endothelial lies in the generation of artificial blood vessels. Several groups have shown that the coverage of artificial blood vessels with OEC seems to be an effective way to overcome problems associated with thrombogenicity [50, 51].

In other approaches, OEC have been combined with biomaterials serving as scaffolds to deliver them to the site of the action. The bio-functionality of such constructs has been assessed in vitro and in vivo. In a recent study the alginate based delivery of OEC from human cord blood has been compared with the application of OEC by bolus injection in an ischemic hindlimb model. Bolus injection of OEC showed no therapeutical effect and resulted in necrosis and foot loss. In contrast, the material based approach successfully induced the vascular reperfusion to normal levels within 40 days and prevented tissue necrosis [52]. This study showed a significant advantage of material based delivery of OEC for therapeutical applications.

Most of the studies described above were performed in matrigel or other gel-like matrices; nevertheless the use of biomaterials depends on the field of application and is another critical element for successful vascularization strategies. In bone tissue engineering, mechanical stability of scaffolding materials is considered as one of the key features. Such scaffolding materials have to support the ingrowth of vessels from the peri-implant tissue and should not interfere with the angiogenic potential of endothelial cells included in the construct.

Furthermore, a series of studies emphasized the close association of bone development and angiogenesis including a mutual information exchange of osteoblasts

and endothelial cells or their respective precursors [53–56] and a beneficial effect in bone formation by strategies improving the vascularization process in general. Although the detailed mechanisms are still unclear, it seems that the co-culture with the primary osteoblasts (pOB) or MSCs provides both a proangiogenic matrix based on components such as collagen and the supply with angiogenic growth factors such as VEGF leading to an angiogenic activation of endothelial cells [57, 58], as well as the inclusion of control mechanisms based on intercellular communication [59–61]. These findings will probably result in new concepts in tissue engineering, such as prevascularization strategies based on the natural interaction of cells as recently addressed by several authors [46, 62–64]. From this point of view, OEC might serve as a potential source of autologous endothelial cells in applications in tissue engineering and regenerative medicine.

At present there are several arguments supporting the potential of OEC for bone tissue engineering applications. First, OEC grow according to our experience on any type of scaffolding materials, which supports the growth and the functionality of endothelial cells in general, so that they could be applied to a series of biomaterials developed for bone tissue engineering. Furthermore, co-cultures of OEC with primary human osteoblasts induce the organization of OEC into prevascular structures characterized by a defined vascular lumen [65] also depicted in Fig. 2. This beneficial effect on the angiogenic activity of OEC in co-cultures with osteoblasts can also be transferred to biomaterial based tissue engineering approaches proven at both levels of investigation in vitro [66] and in vivo [67].

In addition, subcutaneous co-implantation of both cell types, OEC and primary human osteoblasts in a matrigel plugs, improved the formation of actively perfused blood vessels by OEC compared to controls implanting OEC alone [67]. These findings can also be confirmed in applications based on scaffolding materials such as starch polycaprolactone (SPCL) fiber meshes after subcutaneous implantation [67].

Although the detailed mechanisms are still unclear, it seems that the co-culture with the pOB provides both a proangiogenic matrix based on components such as collagen [66] and the supply with angiogenic growth factors such as VEGF and angiopoietin-1 [68], leading to an angiogenic activation of OEC. Another potential effect in co-cultures of endothelial cells and other cells is the stabilization of vascular structures mediated by the matrix components enabling the organization of endothelial cells into matured tube-like structures with the basement proteins collagen type-4 and laminin (Fig. 2), as has been shown before for co-cultures of endothelial cells with fibroblasts [69], smooth muscle cells [36, 42], and MSCs [38].

Similar co-culture approaches by other groups using canine derived OEC and mesenchymal stem cells co-implanted subcutaneously on collagen fiber mesh scaffolds in a nude mouse showed both, a positive effect on the vascularization process as well as on bone formation [70]. Although in this study no direct influence of EPC on the osteogenic differentiation in terms of alkaline phosphatase activity was observed, the improved vascularization per se seems to have a beneficial effect on the bone formation process.

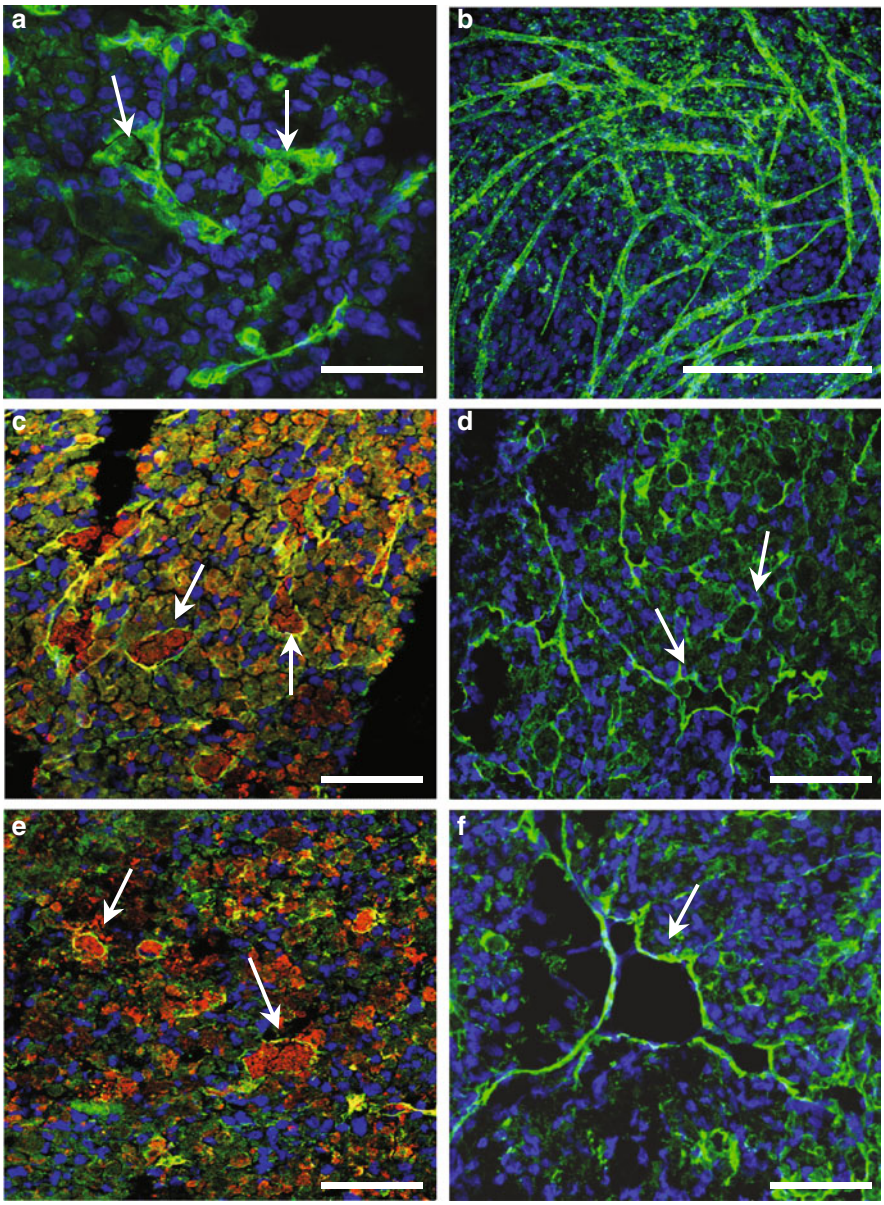


Fig. 2 Co-cultures consisting of outgrowth endothelial cells (OEC) and primary osteoblasts (pOB) stained immunohistochemical for endothelial cell specific marker CD31 after 1 week: (**a**) cross section of constructs derived from rotating cell culture vessels as described in Fuchs et al. 2007 and after 3 weeks, (**b**) two-dimensional culture of co-cultivation. (**c**–**f**) Frozen sections of three-dimensional co-cultures generated in a rotating cell culture vessel system for 3 weeks and stained for laminin (**c**, **d**; *green*) and collagen IV (**e**, **f**; *green*). The endothelial marker vWF is stained in *red* (**c**, **e**). Cell nuclei are counterstained with Hoechst (*blue*). *Scale bars* (**a**–**f**) = 75 μM

Nevertheless, despite their promising outcome, the above-mentioned studies have to be considered as proof of principle studies. Many more studies are needed, focusing on the underlying mechanisms of repair processes and the refinement of protocols to optimize cell isolation efficacies and implantation strategies before these approaches might be applied in clinical practice. In this context, there is also an increasing demand for advanced test systems taking into account the complexity of physiological processes during tissue regeneration. Therefore, a series of co-culture models have been developed over recent years, serving as therapeutical tools, but also as advanced in vitro models to mimic ways of cellular interaction during the repair process and to identify potential targets for therapeutical intervention.

The currently existing concept in tissue engineering is based on the components scaffolds, cells, and growth factors to enhance and accelerate the repair process. Some growth factors are already known as potential candidates for a therapeutical intervention, other potential therapeutical options still have to be identified, for instance by investigation of developmental processes often recapitulated in repair processes in adult life [71–73]. Potential signaling pathways of interest in bone repair are wnt signaling pathways [74, 75] mediating developmental processes by secreted morphogens, as well hedgehog mediated pathways [74] which is involved in the two fundamental processes in bone repair angiogenesis [76] and osteogenesis [77–79]. Sonic hedgehog enhances both processes simultaneously as it has been recently shown in co-cultures of OEC and pOB [68].

7 Self-Endothelialization Strategies

For all the applications based on complex constructs as described above, OEC have to be isolated and expanded in vitro. This is associated with certain risks such as karyotype aberrations during the expansion of OEC [80] in vitro. Last, but not least, in vitro expansion of OEC is time and cost consuming and often incompatible with acute cell therapy of ischemic tissues. Therefore, other strategies aim at a self-endothelialization of functionalized biomaterial surfaces by attracting endothelial progenitor cells from the peripheral bloodstream as recently reviewed in [81]. These approaches include the application of RGD sequences, antibodies, etc. to generate artificial surfaces with a selective affinity for circulating cells with endothelial capacity. Using phage display technologies to identify peptide ligands that bind to OEC but not to human umbilical vein endothelial cells [82], new potential approaches for material modification have been suggested [83]. Other groups used aptamers, single stranded nucleic acids identified by systematic evolution of ligands by exponential enrichment (SELEX) with a high affinity to circulating CD31 positive cells to promote adhesion of cells with endothelial characteristics [84].

8 Conclusions

Endothelial progenitor cells and OEC in particular have raised new hopes as cell sources for proangiogenic therapies. In recent years the picture of therapeutical relevant cell types contained in heterogeneous endothelial progenitor cell cultures became more evident. Nevertheless, although initial ideas concerning a potential application in tissue engineering and regenerative medicine have been developed, a lot of questions need to be answered. These includes aspects of basic endothelial progenitor cell biology as well as the development of new therapeutical concepts by the integration of multidisciplinary research areas.

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Basic Science and Clinical Application of Stem Cells in Veterinary Medicine

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Abstract Stem cells play an important role in veterinary medicine in different ways. Currently several stem cell therapies for animal patients are being developed and some, like the treatment of equine tendinopathies with mesenchymal stem cells (MSCs), have already successfully entered the market. Moreover, animal models are widely used to study the properties and potential of stem cells for possible future applications in human medicine. Therefore, in the young and emerging field of stem cell research, human and veterinary medicine are intrinsically tied to one another. Many of the pioneering innovations in the field of stem cell research are achieved by cooperating teams of human and veterinary medical scientists.

Embryonic stem (ES) cell research, for instance, is mainly performed in animals. Key feature of ES cells is their potential to contribute to any tissue type of the body (Reed and Johnson, J Cell Physiol 215:329–336, 2008). ES cells are capable of self-renewal and thus have the inherent potential for exceptionally prolonged culture (up to 1–2 years). So far, ES cells have been recovered and maintained from non-human primate, mouse (Fortier, Vet Surg 34:415–423, 2005) and horse blastocysts (Guest and Allen, Stem Cells Dev 16:789–796, 2007). In addition, bovine ES cells have been grown in primary culture and there are several reports of ES cells derived from mink, rat, rabbit, chicken and pigs (Fortier, Vet Surg 34:415–423, 2005). However, clinical applications of ES cells are not possible yet, due to their in vivo teratogenic degeneration. The potential to form a teratoma consisting of tissues from all three germ lines even serves as a definitive in vivo test for ES cells.

Stem cells obtained from any postnatal organism are defined as adult stem cells. Adult haematopoietic and MSCs, which can easily be recovered from extra embryonic or adult tissues, possess a more limited plasticity than their embryonic counterparts (Reed and Johnson, J Cell Physiol 215:329–336, 2008). It is believed that these

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stem cells serve as cell source to maintain tissue and organ mass during normal cell turnover in adult individuals. Therefore, the focus of attention in veterinary science is currently drawn to adult stem cells and their potential in regenerative medicine. Also experience gained from the treatment of animal patients provides valuable information for human medicine and serves as precursor to future stem cell use in human medicine.

Compared to human medicine, haematopoietic stem cells only play a minor role in veterinary medicine because medical conditions requiring myeloablative chemotherapy followed by haematopoietic stem cell induced recovery of the immune system are relatively rare and usually not being treated for monetary as well as animal welfare reasons.

In contrast, regenerative medicine utilising MSCs for the treatment of acute injuries as well as chronic disorders is gradually turning into clinical routine. Therefore, MSCs from either extra embryonic or adult tissues are in the focus of attention in veterinary medicine and research. Hence the purpose of this chapter is to offer an overview on basic science and clinical application of MSCs in veterinary medicine.

Keywords Animal models, Clinical stem cell applications, Embryonic stem cells, Immunogenicity, Induced pluripotent stem cells, Mesenchymal stem cells, Regenerative medicine, Stem cell sources, Veterinary medicine

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1 Basic Research: Origin, Functionality and Capacities of Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a population of undifferentiated multipotent cells isolated from adult tissue (e.g. bone marrow or fat), with the capacity to differentiate into mesodermal lineages such as bone, cartilage, fat, and muscle tissue [1–4] and the ability of self-renewal through replication [5].

MSCs participate in tissue regeneration by two distinct mechanisms. They directly contribute to tissue repair by differentiating into specific cellular phenotypes such as tendon or ligament fibroblasts. Of equal importance to the direct differentiation and production of matrix is the production of bioactive proteins by adult stem cells. These factors include various growth factors, anti-apoptotic factors and chemotactic agents that have profound effects on the local cellular dynamics, producing anabolic effects, stimulating neovascularisation, and recruiting additional stem cells to the site of injury. Recruited stem cells may in turn differentiate and/or produce additional biologically active peptides [6].

MSCs can be isolated and expanded with high efficiency (Fig. 1) and induced to differentiate into multiple lineages under defined culture conditions in vitro [2, 7]. MSCs are typically spindle shaped resembling fibroblasts [8].

Due to the lack of specific MSC markers which would allow an exclusive definition of cells as completely undifferentiated stem cells or as lineage committed cells [5], MSCs are identified through their ability to differentiate into multiple lineages, their property to adhere to plastic in vitro and, in human medicine, through a combination of positive expression (CD 105, CD 73, CD 90) or distinct lack (CD 34, CD 45) of typical cell surface markers [9]. However, in veterinary medicine the characterization of stem cells is a bit more difficult because most of the commonly available cell surface markers do not cross react with the animal cells. Thus it is not clear if results indicating a lack of specific surface markers are based on a true lack of these markers or if the human directed markers simply do not cross-react with animal cells [10]. Currently, adherence to plastic and trilineage differentiation potential are the only way to identify MSCs in veterinary medicine [11].

Within the last few years the name "mesenchymal stem cells" has been used very generally for any sort of mesenchymal progenitor cells. Recently the term "mesenchymal stem cells" has been reviewed by the International Society of Cellular Therapy who suggested to rather use the name "multipotent mesenchymal stromal cells" in order to ensure an accurate denomination in scientific literature. The term "mesenchymal stem cells" should be exclusively used for cells with proven in vivo potential of long-term survival with self-renewal ability and the capacity to repopulate multilineage tissue. A precise nomenclature of cell populations will enable a much more accurate comparison of results from different investigators [11]. This is of particular importance in veterinary medicine since differences between species always need to be considered. However, this distinction has not yet been generally accepted and therefore the term used by each author has been maintained in this article.

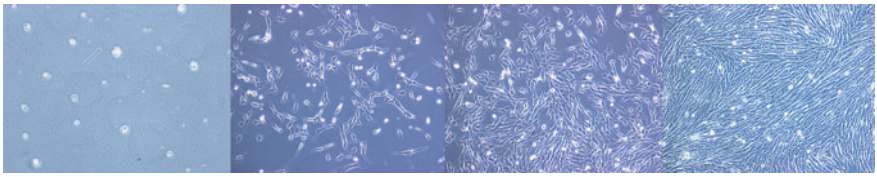

Fig. 1 Different stages of MSC culture (immediately after seeding, 30%, 70% and 100% confluency)

2 Stem Cell Sources

In animals, and in humans, a lot of different tissues representing potential sources of adult stem cells have been identified. Currently, bone marrow (BM) is certainly the best researched source. Even though a lot of alternative sources of MSCs have been described and investigated, bone marrow is still the most commonly used source to recover stem cells [12, 13].

In veterinary medicine not only the potential stem cell yield is important for the definition of a good cell source but also species specific difficulties regarding the process of stem cell recovery and the associated costs certainly need to be taken into consideration. Therefore, the practicability of different stem cell sources in veterinary clinical practice does not always correlate with the theoretically best source from a cell yield and quality perspective. Nevertheless it has repeatedly been described that there are significant differences in MSCs from different tissue sources and that findings from one species cannot necessarily be extrapolated to another [14].

In future the identification of the optimal mesenchymal tissue as a source of MSCs depending on the intended use and the animal species will play an important role. Also the cell source to treat best a particular clinical condition is yet to be discovered.

Until proven otherwise, bone marrow remains the most reliable source of progenitor cells in veterinary medicine [15].

2.1 Bone Marrow

Isolation of bone marrow derived MSCs, also known as marrow stromal cells or mesenchymal stromal cells [16, 17], has been described in several animal species [18] such as rabbits, mice and rats, horses [3], dogs [9, 19], cats [20], pigs [21] and cattle [22].

Bone marrow contains not only mesenchymal fibroblast-like stem cells but also a high amount of haematopoietic stem cells. The MSCs can easily be separated from the haematopoietic cell fraction by culture and adherence to plastic dishes.

Primary bone marrow derived nucleated cells vary in morphology and include large widespread, occasionally multi-nucleated cells and spindle shaped mononuclear cells. With subsequent passages this degree of heterogeneity decreases and the small spindle shaped fibroblast like cells predominate [16]. In contrast, Giovannini et al. [18] reported that MSCs obtained from equine bone marrow always display the same fibroblastic morphology.

Opinions regarding the quality of bone marrow as MSC source are controversial, as there seem to be significant differences between different species.

Even though it is reported that in mature individuals bone marrow is generally the richest source of stem cells presently known in humans as well as animals [15], Yoshimura et al. [14] found that the colony formation rate of primary bone marrow derived MSCs in rats and humans seems to be lower than that of MSC derived from other mesenchymal tissues. The number of primary colonies per nucleated cell from synovium, periosteum, adipose and muscle tissue seems to be much higher [14].

An advantage of bone marrow derived stem cells is that they can easily be passaged many times and over long time periods [15, 23]. They also show good differentiation potential. However, Vidal et al. [16] showed that adipogenesis of equine bone marrow MSC is satisfying only after adding 5% rabbit serum to the culture medium.

In bone marrow the achievable MSC yield also varies between different species: canine and feline BM-derived MSC frequency, for example, is about 1 in 2.5×10^4 and 1 in 3.8×10^5 respectively, whereas in murine bone marrow MSC frequencies range between 1 in 10.8×10^3 and 1 in 3.45×10^4. In horses an MSC frequency of 1 in 4.2×10^3 is reported [16].

Ultimately, bone marrow is not an optimal source for MSC, because the collection procedure is painful and contains a non-negligible risk of haemorrhage and infection as well as sepsis [18]. Additionally, in veterinary medicine there is a relatively high safety risk for the veterinarian due to the collection modality in particular collecting bone marrow from horses, which is either performed from the patient's sternum or tuber coxae (Fig. 2 and 3).

Fig. 2 Bone marrow collection from a horse's sternum

Fig. 3 Bone marrow collection from a horse's tuber coxae

Bone marrow collection in a horse is usually conducted under standing sedation. If bone marrow is collected from the tuber coxae the veterinarian is standing right next to the patients hind leg and can easily be kicked. If the bone marrow is aspirated from the horse's sternum the operating veterinarian is kneeling under the sedated horse and can therefore easily be kicked as well. Some horses also tend to fall down either due to the relatively heavy sedation or in an attempt to hinder the veterinarian from puncturing their sternum. This is associated with some risk for the veterinarian (who might get caught underneath the horse) and the patient itself, because the needle might be pushed further into the sternum when the horse's chest touches the ground.

In rodents, who play a major role in animal experiments, using MSCs we are facing a different problem, as the collection and isolation of sufficient amounts of bone marrow MSCs is quite difficult due to the small body size of the animals, resulting in a limitation of the feasible number of in vivo experiments [14].

2.2 Peripheral Blood

Peripheral blood (PB) compared to bone marrow and solid tissue displays a safe and virtually pain-free source for stem cell recovery.

Unfortunately, isolation and proliferation of fibroblast like cells from PB requires very sophisticated techniques. Only in mice, guinea pigs and rabbits can standard isolation protocols as known for bone marrow derived MSCs be used [15]. In horses, Smith et al. [24] processed blood samples using a slightly modified method as for equine bone marrow, but were not able to isolate fibroblastoid cells.

This is in agreement with data obtained in human medicine and in canines, where it was reported that the isolation and propagation of PB derived fibroblast like cells from mature individuals is difficult [25]. A study on equine PB derived stem cells performed by Koerner et al. [15] revealed that only 36.4% of the samples

gave rise to fibroblastoid cells. In these samples only 1–5 cell colonies were observed after 14 days. Giovannini et al. [18] used more sophisticated isolation techniques and were able to isolate successfully fibroblast like cells from 8 out of 12 PB samples (75% success rate).

Both Giovannini et al. [18] and Koerner et al. [15] found cells of different morphologies in the initial culture. One group of colony forming units (CFUs) consisted of cells with a more fibroblastic shape whereas other cells in other CFUs showed a more distinct polygonal morphology. Nonetheless, similar to bone marrow, the morphologic differences observed in the initial culture were lost after the first passage [18].

Another interesting finding was that, as a consequence of continued passaging, equine PB derived stem cells either stopped proliferating or grew in a side by side primary structure and a net-shaped secondary structure. After about five passages the proliferation capacities of PB progenitors seem to cease [15].

In addition, the differentiation potential of blood derived MSCs seems to be inferior compared to other stem cell sources as Koerner et al. [15] were not able to induce chondrogenic differentiation. In contrast, Giovannini et al. [18] were able to show that equine PB derived fibroblast like cells do have the potential to differentiate into the three common mesodermal lineages but only under specially optimised differentiation conditions.

Moreover, equine PB progenitor cells proved very sensitive to trypsinisation as well as cryostorage in liquid nitrogen and thawing [15], which significantly alters their usefulness in regenerative medicine in the long run. In addition, the limited differentiation potential observed by Koerner et al. [15] as well as the slower differentiation response towards cartilage and bone observed by Giovannini et al. [18] indicate that PB derived fibroblastoid cells might not be the same cells as bone marrow MSCs.

2.3 Umbilical Cord Blood

Compared to human medicine, umbilical cord blood (UCB) collection has only recently moved into the focus of interest in veterinary medicine. Therefore, experience in this field is still very limited and mainly restricted to the horse. According to our knowledge, UCB banking is only commercially available for horses.

In equine medicine it was shown that UCB can be collected without complications for either the foal or the mare at the time of foaling (Fig. 4a, b, c). The only downside is that UCB can only be collected if a veterinarian or somebody else who is capable of drawing blood is present at the birth, which often is not the case.

UCB stem cells show slightly different characteristics when compared to other adult stem cells. They are proven to differentiate into cell types characteristic for mesodermal and endodermal origins. Their ability to form hepatocytes suggests that UCB derived cells may be more plastic than MSCs derived from other adult

Fig. 4 (a, b, c): Collection of equine umbilical cord blood

tissues. In addition, Oct4 – a characteristic embryonic stem (ES) cell marker protein – was identified in over 90% of the cell nuclei of equine UCB derived cells [26].

Compared to other cell sources like bone marrow and fat, the achievable MSC yield from UCB is relatively low. Only in four out of seven UCB samples could colonies with MSC morphology be detected. Additionally, and similar to humans, the achievable number of primary cultures (between one and five) is relatively low [13]. This was also confirmed by Reed and Johnson [26] who reported that the yield of adherent cells was poor.

Similar to PB and bone marrow, the morphology of cultured UCB cells varies [13, 26]. The recovered cell population was heterogeneous with typical slender and elongated spindle shaped cells and cell clusters of cuboidal cells with shorter cytoplasmatic extensions. It is not yet clear why undifferentiated cells show different morphology and if they display different stem cell phenotypes [13, 26].

Equine UCB derived MSCs can be successfully differentiated towards the osteogenic, chondrogenic and adipogenic cell fate [13, 26]. However, formation of adipogenic [13, 26] and muscle cells was not efficient [26]. It was only possible to induce adipogenic differentiation after adding rabbit serum to the culture media [13].

In contrast to PB derived MSCs, it was reported that cryo preservation, thawing and post thawing expansion had no negative influence on either cell morphology, proliferation potential or differentiation capacity of UCB derived MSCs [13].

Based on that knowledge, and similar to human medicine, commercial storage of equine UCB derived stem cells for later use for autologous stem cell transplantation is available and might offer the same potential as in humans [13].

2.4 Stem Cell Recovery from Solid Mesenchymal Tissues

Sakaguchi et al. [27] reported that the colony forming efficiency of suspended cells from solid mesenchymal tissues following collagenase digestion is about 100-fold higher than that of bone marrow. This was confirmed by Yoshimura et al. [14] who reported that the yield and proliferation capacity of MSCs from solid tissues was much better than from bone marrow.

2.5 Adipose Tissue

Together with bone marrow, fat, which is more abundant and more easily accessible than bone marrow [28] (Fig. 5), is the most frequently used stem cell source in veterinary medicine. Unfortunately, evidence that fat derived stem cells are qualitatively and quantitatively comparable to bone marrow derived stem cells is still missing. Regardless of that, stem cell therapies for animals (dogs and horses) using fat derived cells for the treatment of osteoarthritis (OA) as well as tendon and ligament injuries are already on the market. However, commercially available "stem cell therapies" using fat derived stem cells cannot always be referred to as real stem cell therapy. The MSC rate in the nucleated cell fraction of fat is very low and only culture purification and expansion leads to a sufficient MSC yield. Hence the application of the nucleated cell fraction without prior cell purification and expansion cannot be referred to as true stem cell therapy.

However, fat might be a useful alternative to bone marrow because it can easily be obtained from subcutaneous tissue which is less invasive than a bone marrow aspiration [13] and therefore associated with less risk and pain to the patient. In addition it is usually available in large amounts [29].

Recently studies comparing MSCs from fat and bone marrow regarding their quality and quantity performing FACS- and PCR-analysis as well as differentiation and proliferation experiments were carried out in horses. It was confirmed that the differentiation potential of fat derived stem cells is similar to bone marrow derived MSCs [30, 31]. Comparing the yield of adherent cells, growth kinetics, cell senescence and efficiency of gene transduction between MSCs from bone marrow and MSCs from adipose tissue, it has been reported that there is no difference between the cells derived from these two sources [32]. Interestingly, more recently Conrad et al. [30] and Mundle et al. [31] in contrast demonstrated that MSCs derived from fat show a twofold faster proliferation compared to bone marrow MSCs in vitro. This was lately confirmed by Dahlgren [6] who reported about a higher frequency of stem cells in fat compared to bone marrow (2% vs 0.002%) with an average cell yield of 450.000 per gram of fat and a higher proliferation rate. Also Reich et al. [28] found a shorter population doubling time and faster migration into an artificial wound area when comparing fat with bone marrow derived stem cells.

Interestingly, this seems to be in contrast to humans as Sakaguchi et al. [27] reported that MSCs derived from human adipose tissue had a lower proliferation potential than other mesenchymal tissue derived MSCs. On the other hand, Kern et al. [33] demonstrated a higher proliferation potential of human adipose tissue derived MSCs. Regarding the number of colony-forming units [33] and population doublings [29], fat is reported to be a better source of progenitor cells as well.

Comparing the MSC differentiation potential from different sources in vitro and in vivo, MSCs from adipose tissue of rodents seem to have the lowest chondrogenic potential [14, 34] based on their reduced expression of bone morphogenetic protein (BMP)-2, -4, -6 and lack of TGF-β receptors, which was also found in human adipose

Fig. 5 Fat recovery location in horses [145]

tissue [35]. Also, canine adipose derived MSCs seem to have a lower chondrogenic differentiation potential compared to bone marrow MSCs whereas the osteogenic potential appeared to be comparable [28].

As expected, the adipogenic differentiation potential is obviously higher in adipose derived MSCs than in MSCs from other sources [14].

2.6 Umbilical Cord

The umbilical cord (UC) matrix or Wharton's jelly of humans and animals is reported to be a particularly rich source of very young MSCs with high proliferation ability (Fig. 6). The gelatinous connective tissue of the umbilical cord consists of myofibroblast like stromal cells, collagen fibres and proteoglycans [10, 36].

Umbilical cord MSCs have so far only played a minor role in veterinary science and no clinical applications are known to date. However, promising results in human medicine are already available and have prompted the first veterinary medical in vitro studies (mainly in equine medicine), which might serve as basis for future studies and possible first clinical applications.

Stem cell isolation from umbilical cord tissue (Wharton's jelly) is easy and practicable as a simple collagenase digestion of small, blood vessel free matrix pieces is performed [36]. The average number of cells and CFUs calculated at the end of the primary culture and the population doubling as well as fold increase of umbilical cord derived stem cells are reasonably high [36].

In addition, they can be cryogenically stored and brought back into culture without obvious changes regarding their growth or phenotypic characteristics [10, 36].

Umbilical cord matrix cells show functional similarities to MSCs from other sources [10, 36]: It could be shown that the fibroblast like cells can differentiate into the three major mesenchymal lineages bone, cartilage and fat [36]. Interestingly, again three morphological types of cells in the primary culture were observed

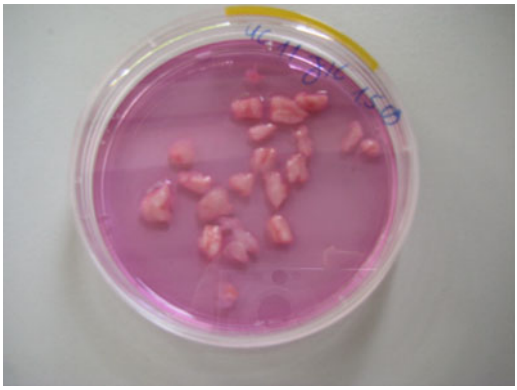

Fig. 6 Isolation of MSC from umbilical cord tissue via migration onto culture dish

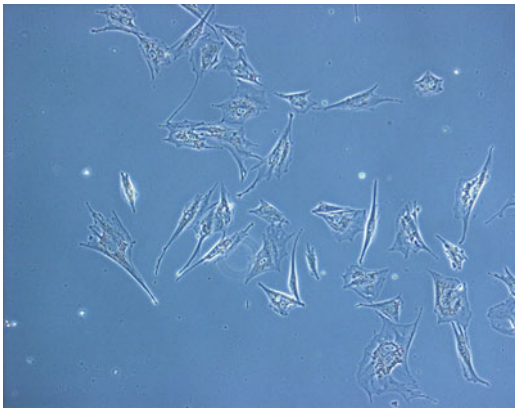

Fig. 7 Different MSC morphology in primary culture

(Fig. 7): Large and occasionally multi-nucleated cells, small, spindle-shaped, mononucleated cells and stellate cells. The large and occasionally multi-nucleated cells disappeared after the first passage and the small, spindle-shaped fibroblastoid cells predominated [36].

It was reported that UC MSCs express embryonic marker proteins like Oct-4, SSEA-4 and c-Kit [10, 36]. Therefore, it is hypothesised that they represent a primitive phenotype between embryonic and adult stem cells [10, 36]. This hypothesis was supported by the findings of Mitchell et al. [37] who demonstrated that cells isolated from porcine umbilical cord matrix are able to differentiate into cells that morphologically resemble neurons and express proteins specific for neurons and glia cells. In addition, Weiss et al. [38] showed that porcine umbilical cord matrix cells express markers of mature neurons when transplanted into rat brain. Rat umbilical cord matrix cells show similar properties and equine umbilical cord matrix cells were also demonstrated to adopt a morphology typical for neurons with axon and dendrite like processes upon culture in the right medium [10]. These findings confirm that MSCs from extraembryonic tissues are able to differentiate

into cells from distinct germ layers like mesoderm and ectoderm. Another indication is that a large subset of the cultured umbilical cord matrix cells remain in the quiescent state which is related to self renewal ability [10].

Stem cells from extraembryonic tissues are furthermore expected to express low immunogenicity and may, therefore, potentially serve as allogeneic donor cells in the future [10]. However, trials proving the allogeneic applicability of extra-embryonic tissue derived stem cells in regenerative medicine are still missing. Hoynowski et al. [10] evaluated the expression of markers related to immunogenicity such as HLA-ABC, HLA-1AG and MHC-11. Unfortunately, they were unable to confirm whether the lack of significant expression was truly negative or if equine cells simply do not cross react with the reagents developed for human cells.

All these findings indicate that the umbilical cord matrix seems to be a good alternative to bone marrow. MSCs from the umbilical cord can be collected in a non-invasive manner at birth and stored for future use [10, 36]. The only downside is that the maternal perineum and the delivery environment are certainly not sterile [36], particularly referring to animals. Therefore, a sterile collection is challenging and only samples cultured in a medium containing a relatively high amount of antibiotics can be considered suitable for experiments and clinical applications.

2.7 Synovial Membrane

The synovial membrane (synovium) lines the inside of joint cavities, bursae and tendon sheaths and regulates the content of the synovial fluid which is contained in these cavities. The first successful extraction of MSCs from the synovium was performed in humans by De Bari et al. [39]. According to Fan et al. [12] and Yoshimura et al. [14], stem cells from the synovial membrane of humans and rats excel other sources of MSCs in higher ability of proliferation and superiority in chondrogenesis and adipogenesis. The achievable colony number per nucleated cells was reported to be 100-fold higher than that of bone marrow derived rat MSCs. Compared to other MSC sources, synovium-derived stem cells were also highest in colony forming efficiency, fold increase and growth kinetics [14]. On the other hand, they seem to be inferior in osteogenic capability compared to periosteal-derived MSCs and bone marrow derived, but still superior in comparison to fat and muscle derived stem cells [12, 27].

In addition to the in vitro results, multiple reasons why it is assumed that synovium derived MSCs are especially superior in chondrogenesis compared to MSCs from other sources were reported.

Synovium derived MSC have a higher hyaluronan receptor expression and express enzymes involved in hyaluronan synthesis, the synovial membrane is further believed to contribute to repair of partial thickness cartilage defects [12] and cartilage can be formed in pathological synovial tissue (synovial chondromatosis) and synovial pannus of rheumatoid arthritic knee joints [12, 14].

Moreover, synovium and cartilage originate from a common source of progenitor cells and synovial tissue expresses a variety of cartilage specific markers [12].

A very interesting finding in particular for the treatment of tendon injuries is that synovium derived MSCs can serve as hyaluronic acid blasters, avoiding adhesion, a common complication in tendon injuries that can lead to scar tissue formation [12].

Although they can also be extracted from pathological synovium [12] and only a minimal amount of synovial tissue is necessary for the extraction of a reportedly high amount of MSCs, and in spite of the high regeneration rate of the synovium which leads one to expect few complications at the donor site [12, 27], the cell recovery process by arthroscopy requires general anaesthesia and is therefore associated with a relatively high risk for the patient, especially in large animals. Furthermore, it would also be expensive. In addition, preparation of the synovial tissue for stem cell extraction is not easy. It was reported that the separation of the subsynovial tissue from the synovial tissue – which is required in order to obtain homogenous cell culture – is difficult. Another question regarding the quality of MSCs from the synovium that needs to be addressed is the fact that some of them seem to retain their fibroblastic characteristics even after differentiation induction [12]. Therefore, synovium might be a practicable MSC source in human but not necessarily in veterinary medicine.

2.8 Periodontal Ligament

In equine orthopaedics, MSCs have attracted much notice because of promising results of MSC treatments of superficial digital flexor tendonitis. However, recovered tendons have inferior biomechanical properties compared to healthy tendons. Consequently, a source of MSCs is needed which guarantees a high tenogenic differentiation capacity. In this regard, the periodontal ligament (PDL) earns much attention. Under physiological conditions, the equine PDL combines two remarkable characteristics. It withstands high biomechanical strains presenting characteristics similar to a tendon, and at the same time it possesses a high regenerative capacity [40].

The periodontial ligament is situated between the tooth and the jaw bone and is part of the complex that keeps a tooth in place in its alveolar cavity. In veterinary medicine the PDL as source for MSCs was only described in rodents and horses.

In order to compensate for rapid surface attrition the hypsodont equine cheek tooth erupts continuously which is inevitably associated with permanent remodelling of the periodontium. Therefore, the periodontium shows a rapid cell turnover compared to other soft connective tissues. The equine PDL for example shows a proliferation index of 1–3% [41]. In addition, the functional requirements of the PDL depend on ample capacity for dynamic changes regarding tissue synthesis remodelling and repair. This is only possible because of the inherent capacity of the periodontial cells to differentiate into osteoblasts, collagen-forming fibroblasts or cementoblast [42, 43].

It has been proposed that the periodontium comprises a population of undifferentiated progenitor cells which migrate either towards the cementum to differentiate into cementoblasts or towards the alveolar bone to become osteoblasts [41]. Gould [44] and McCulloch [42] showed typical characteristics of these cells in mice which strongly indicate that they indeed are stem cells. A population of progenitor cells which may be stem cells was also found in the paravascular sites of the mouse molar PDL [42].

Only recently these suggestions were confirmed. MSCs in the periodontial ligament of sheep and pigs – which were able to differentiate into a large variety of cell lineages in vitro – were detected [45, 46]. At the same time it was shown that there seems to be a considerable difference between PDL derived and bone marrow derived MSCs based on their higher expression of tenocyte specific transcription factors [47]. Periodontal cells also showed significantly higher capacities for self-regeneration, i.e. number of CFUs, than cells from the subcutis, whereas the population doubling time of subcutaneous cells seems to be significantly faster than those of PDL cells. All cells showed osteogenic and adipogenic differentiation. Marker mRNA for chondrogenic differentiation (Aggrecan, Collagen 2, COMP) was highly expressed by cells from the middle and apical areas of the PDL. In contrast, in subcutaneous cells and PDL cells from the subgingival area the expression of chondrogenic marker mRNA was limited to Aggrecan and COMP. The equine PDL contains cell populations that exhibit typical properties defined for MSCs. Cells from the apical and the middle areas showed higher differentiation capacities than subgingival cells and subcutaneous cells [40].

Equine PDL cells might be a promising source for MSC-therapies in equine musculoskeletal disorders [40]. However, the PDL contains only a small number of progenitor cells [44], suggesting that the role as practicable MSC source in regenerative medicine needs to be questioned.

2.9 Skin

MSC derived from skin would be very easily accessible (regardless of the species) with low costs and a low risk for both the patient and the veterinarian.

In juvenile and adult rodents it was shown that stem cells can be isolated from the dermis. Interestingly, these cells seem to be able to differentiate into neuroectodermal and mesenchymal lineages, including neurons, glia, smooth muscle cells and adipocytes. Based on these findings, rodent dermis derived stem cells are distinct from MSCs. They can be passaged for at least one year without losing their differentiation capacity and therefore probably represent a novel multipotent adult stem cell type. They also clearly differ from adherent bone marrow derived stem cells in the way that they require different growth factors to proliferate and their selective ability to express proteins typical for neuronal precursors, as well as their morphology and habit to grow in spheres. It is suggested that these precursor cells represent a novel multipotent adult stem cell capable of generating cells from

more than one embryonic lineage [48]. However, further investigations will be necessary to confirm these findings.

2.10 Other Potential MSC Sources

Other potential sources of MSCs are muscle, brain [15, 49], synovial fluid [50], tendon [51] or periosteum [14]. Although rodent models for these tissues exist, the practicable isolation of these cells as a common stem cell type used in regenerative medicine is doubtful.

Regarding the differentiation ability, it is remarkable that MSCs derived from muscle produce only tiny pellets after chondrogenic differentiation [34]. However, they show a good calcification potential after osteogenic differentiation [14] as well as an easy differentiation to adipocytes [52]. According to Koga et al. [34] muscle-derived MSCs have a higher proliferation potential than other stem cells [34].

Although the isolation of brain-derived MSCs after enzymatic digestion of the whole brain is not a practicable way to harvest MSCs for tissue engineering purposes, it is interesting to see that this stem cell type has a less efficient adipogenic proliferation potential [52].

MSCs isolated from the cambium layer of the periosteum have a high chondrogenic proliferation potential which results in a greater production of cartilage matrix. Regarding the calcification ability, it is not surprising that the periosteum-derived MSCs have a high osteogenic proliferation potential [14].

3 Immunogenicity

The immunogenicity of adult MSCs is not completely understood. MSCs are said to be hypo-immunogenic and to suppress T-cell activity and dendritic cell function [8, 11] in humans and animal models [8].

Normally, allogeneic cells would be rejected by immune response. Surprisingly, immunologists found that MSCs do not seem to obey the normal rules of allogeneic rejection. Evidence indicates that the use of mismatched MSCs does not provoke a proliferative T-cell response, thus suggesting an immunosuppressive potential [8].

Krampera et al. [53] found that murine MSCs lack MHC class II and inhibit T-cell activity. Furthermore, Tse et al. [54] showed in humans that MSCs do not elicit allogeneic T-cell response even when MHC class II was upregulated. It was also reported that allogeneic baboon MSCs suppress lymphocyte activity in vitro and prolong graft survival, indicating the anti inflammatory and pro healing effect of MSCs which was later confirmed by Di Nicola et al. [55], Tse et al. [54] and Krampera et al. [53].

MSCs appear to evade allogeneic rejection by being hypoimmunogenic, interfering with maturation and function of dendritic cells, modulating CD4 and CD8

T-cell phenotype and response as well as natural killer cell activity and creating an immunosuppressive milieu based on secretion of a variety of soluble factors [8].

Recently, allogeneic MSC transplantations were carried out in rabbits and horses, showing no significant difference compared to autologous transplants.

Autologous and allogeneic bone marrow derived MSCs in a fibrin carrier were implanted into rabbit Achilles tendons. In accordance with Guest et al. [56] it was found that the distribution of inflammatory cells was similar in the allogeneic and the autologous group. No apparent immune reaction such as lymphocyte infiltration associated with the allogeneic transplantation was observed. Viable allogeneic MSCs were detectable at 8 weeks post implantation [57].

In another study, autologous and allogeneic green fluorescent protein (GFP) labelled mesenchymal progenitor cells (MPCs) were injected into artificially created superficial digital flexor tendon (SDFT) lesions in horses. A very interesting finding was that no differences in either the number or distribution of autologous and allogeneic cells as well as in the density of leukocytes at the respective injection sites were observed. Injection of allogeneic MSCs did not lead to any immune response from the host. Neither external nor histological signs of increased inflammation were found compared to the autologous injection site [56].

Therefore, MSCs really seem to be immunoprivileged and one could possibly provide a readily available source of allogeneic MSCs for regenerative medicine purposes at least in veterinary medicine. What needs to be kept in mind, though, is the inherent risk of disease transmission from donor to recipient [5]. However, some day regenerative veterinary medicine might be able to rely on allogeneic cells to repair or replace tissue [8].

4 Clinical Applications of Stem Cells in Veterinary Medicine

Stem cell therapy in veterinary medicine is gradually turning into clinical reality. Especially in equine orthopaedics and small animals stem cell treatments are being commercially offered (Fig. 8).

It was demonstrated that special chemokine receptors enable MSCs to respond to signals produced by damaged tissues [56]. As a response to these signals MSCs migrate into the damaged tissue and seem to induce regeneration of the respective tissue. Therefore, a lot of hope and research emphasis is put into the newly evolving field of human as well as veterinary regenerative medicine.

However, not every treatment that is being advertised as stem cell therapy is actually what it promises to be.

In many cases direct injection of crude bone marrow or nucleated cells isolated from fat without further culture expansion is performed. This treatment is often wrongly referred to as stem cell therapy, which might lead to misunderstandings [58]. As a matter of fact the transplant mainly consists of nucleated cells rather than actual stem cells.

Herthel [59] for example reported that direct bone marrow injection for the treatment of suspensory ligament (SL) injuries led to significantly better results and a decreased reinjury rate – 92% of the bone marrow treated horses went back to work compared to 84.8% that did not become sound or did not go back to work that had received conventional treatment.

For multiple reasons the success of this treatment is questionable.

First of all, bone marrow contains only a small number of actual stem cells. Therefore, the treatment cannot be referred to as actual stem cell treatment [5, 24, 58]. Convincing studies show that only about 0.001–0.01% of mononuclear cells isolated from bone marrow aspirate using Ficoll density gradient are MSCs. Hence the number of MSCs in crude bone marrow would actually be even less than 0.001–0.01%. In horses under 5 years of age it was shown that only 1–2 $\times$ 10^5 adherent cells can be obtained from 10 mL of bone marrow aspirate after 3 days in culture [5].

Second, crude bone marrow might contain bone spicules and fat cells which can be deleterious to tissue regeneration [24].

In contrast, injection of in vitro expanded MSCs provides a larger number of MSCs than endogenously available or delivered by direct bone marrow injection and additionally avoids the risk of adverse effects of other bone marrow constituents [24].

Therefore, it is important to interpret cautiously results from studies using stem cell treatment because the term "stem cell therapy" is not always used in the correct way and might be misleading.

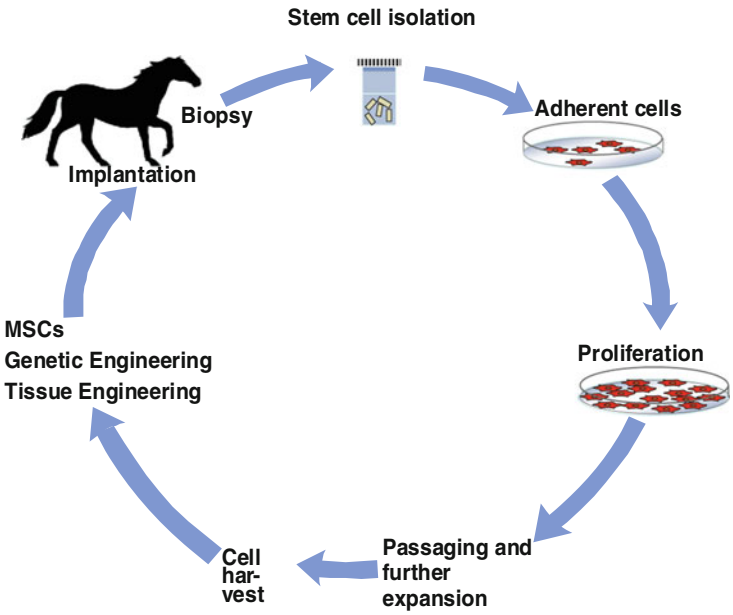

Fig. 8 MSC therapy principle

4.1 Tendon Injuries

In addition to in vitro studies and small animal experiments, it is certainly the horse that veterinary research is focussed on concerning stem cell treatment of tendon injuries.

Tendon injuries are a frequently occurring problem in the equine athlete (Fig. 9). Due to the equine quadruped-specific anatomy characterised by the proximally located muscles and the distally located long SDFT, deep digital flexor tendon (DDFT) and SL in combination with the hyper-extended metacarpophalangeal joint, equine tendons and ligaments are exposed to enormous forces during athletic workout. Maximal strains in the SDFT are reported to be at 16%, which comes up to the functional limit, during galloping in thoroughbreds [60].

After suffering a clinical injury, a short inflammatory phase is observed in the tendon, followed by the creation of fibrous scar tissue. This scar tissue lacks elasticity compared to healthy tendon and therefore the risk of re-injury is high [61]. Outcomes following conventional treatment regimes unfortunately are rather poor [62]. As stem cell therapy encourages the regeneration of functional tendon tissue rather than scar tissue, it is expected to reduce re-injury rates [56]. Two possible theories regarding the effect of stem cells are discussed. One possibility is that they differentiate into tenocytes within the tendon environment and support healing via collagen production and remodelling activities. The second possibility is that the injected cells supply growth factors rather than differentiate terminally into the required tissue [57, 60].

It is proposed that the introduction of MSCs into the tissue which contains the required cell type, in addition to the adequate mechanical environment, provides the best stimulus for appropriate differentiation [24]. In case of stem cell treatments for tendon or ligament lesions it is suggested that tensional mechanical load is necessary for an optimal formation of organised tendon and ligament matrix [63].

Therefore, equine tendinopathy, with its typical centrally-positioned damage surrounded by relatively intact tendon tissue or at least the thick paratenon offers

Fig. 9 Equine tendinitis of the SDFT (courtesy of Dr. Johannes Edinger)

perfect conditions for stem cell applications (Fig. 10, 11 and 12). Abundant growth factors are also involved in early tendon healing and provide a perfect graft bed for the injected MSCs. However, treatments of other forms of injuries are more problematic mainly because accurate placement of the cells and cell retention is more difficult [24].

In many cases direct intratendinous injection of crude bone marrow to support tendon healing is performed, which was first reported by Herthel [59]. Although the results of this study were favourable compared to conventional treatment, the success of this technique is questionable for multiple reasons which have already been discussed above. In addition, injection of large volumes of bone marrow might even exacerbate the tendon injury, due to disruption of remaining intact tendon tissue [24].

There are two different approaches of stem cell therapy that are clinically used for the treatment of equine tendon disease: one is to apply isolated and expanded bone marrow-derived MSCs, the other is to implant adipose-derived nucleated cell (ADNC) fractions [60] or adipose derived expanded MSC.

The latter technique was tested in a small controlled experimental study with eight horses suffering from collagenase-induced tendinitis. Five days after creation of the SDFT lesions, adipose tissue was harvested from the paraxial caudodorsal gluteal region under standing sedation and local anaesthesia. Collagenase digestion and serial centrifugation was used to isolate and purify the ADNC fractions which were then resuspended in phosphate buffered saline (PBS) solution, in order to be injected only 2 days after adipose tissue harvest and 7 days after the lesions were created. Four horses obtained ADNC-treatment, the others served as control. Ultrasonographic and, 6 weeks later, gross and histologic examination revealed an improvement in structural organisation and a reduction of inflammation in the ADNC-treated tendons compared to the controls. Gene expression for COMP was also significantly increased (concentrations of COMP, a noncollagenous glycoprotein, are positively correlated with ultimate tensile strength and stiffness in equine tendons). However, analysis of collagen revealed no significant differences between the two groups [64]. Unfortunately, although this technique is widely

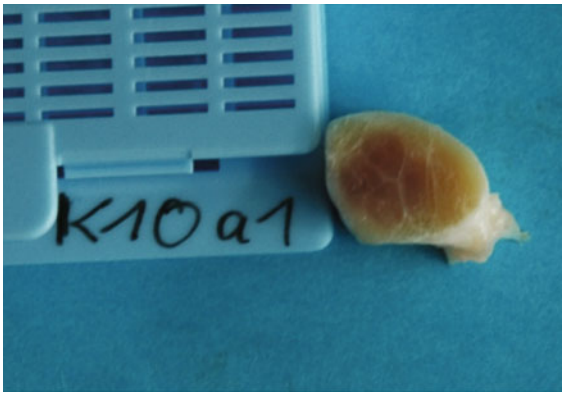

Fig. 10 Core lesion in an equine SDFT

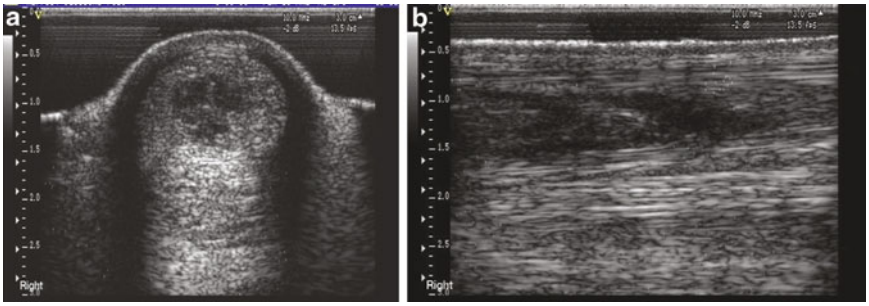

Fig. 11 Sonography of a core lesion in an equine SDFT. (**a**) Transversal view. (**b**) Longitudinal view (courtesy of Prof. Roger Smith)

used in the USA, there seems to be little information concerning the clinical outcomes available so far. Dahlgren [6] reported that a total of 78% of sport horses have returned to their previous level of performance and 69% of race horses have returned to race more than once. Also Leppänen et al. [65] showed some promising results after the application of enriched adipose derived stem cells in treatment of equine tendon and ligament injuries. Significant improvement in ultrasonographic fibre alignment scores and echogenicity scores were found during the follow-ups at 1, 3 and 6 months after the treatment. After a year from the injury 85% of the horses in the recovery population ($n = 31$) were back to competing and 75.9% of all patient owners included in a survey ($n = 44$) reported excellent or good satisfaction, no significant adverse effects being reported [65].

These results are promising indications of good clinical success using the procedure. The full potential of adipose-derived adult stem cell technology will become evident in the coming years [6].

The major advantage of using ADNCs would be the immense reduction of the interval from tissue harvest until cell application which minimises the cost and simplifies laboratory procedures. Studies have revealed that approximately 80% of the cells isolated from human lipid aspirates are multipotent MSCs [64]. However, this has not yet been confirmed for the horse, and therefore this kind of treatment should not be referred to as stem cell therapy in the narrow sense.

Another approach, using BM-derived MSCs, is performed according to a technique reported in [24]. By now, some aspects of this technique have been modified. To name the most considerable ones, first the number of injected cells rose from 500,000 cells mentioned by [24] to approximately 10×10^6 cells [61, 66, 67]. Pacini et al. [66] observed that a cell number of less than 1×10^6 was insufficient for tendon healing. Second, while Smith et al. [24] used fresh autologous plasma to resuspend the cells before injection, nowadays citrated bone marrow supernatant is applied, which has stimulatory effects on the injected cells and, due to the diffusion of the citrate, clots after injection [68]. Nevertheless, there are also other approaches, such as using PBS [67], autologous serum [66] or fibrinogen [69].

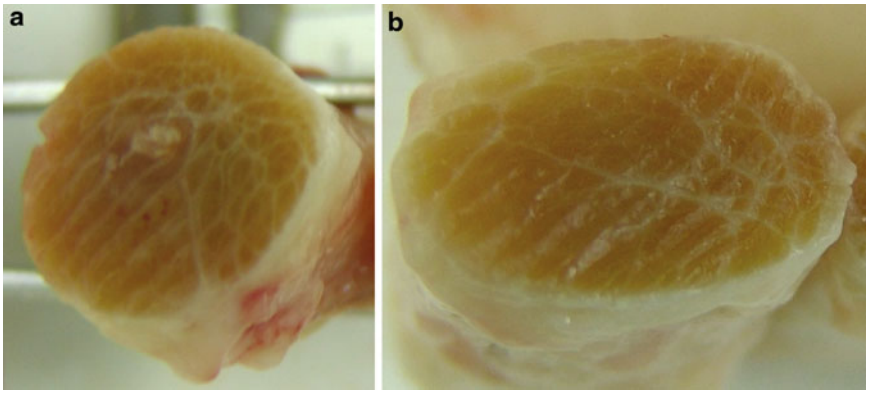

Fig. 12 (**a**) Equine tendon after conservative treatment – obvious scar tissue formation. (**b**) Equine tendon after MSC therapy – no scar tissue formation (courtesy of Prof. Roger Smith)

In a more recent study, eight horses with naturally occurring SDFT injury were used. Autologous bone marrow derived MSCs were expanded in vitro, suspended in citrated bone marrow supernatant and 1×10^7 implanted into the damaged SDFT of four horses under ultrasound guidance. Saline was injected into four controls. Horses received controlled exercise and were euthanised after 6 months. However markers of regeneration in tendon were not identified but a normalisation of biomechanical (reduced stiffness), histological (lower scores) and compositional parameters (lower GAG content) towards those levels in normal (or less injured) tendon could be considerable surrogate markers of regeneration. MSC implantation results in a tissue more like normal matrix rather than fibrous scar tissue formed after natural repair (Fig. 12a, b). MSC-treated SDFT had greater elasticity than saline-treated SDFT ($p < 0.05$). Cross-sectional area of MSC-treated tendons was lower than saline-treated tendons ($p < 0.05$). Histologically, MSC-treated tendons had improved cellularity and organisation scores at the injured site and were comparable to uninjured sites of the treated tendon. In the MSC-treated SDFT, collagen-linked fluorescence was higher and DNA content lower than the saline-treated SDFT ($p < 0.05$). Collagen and GAG content was lower in MSC-treated SDFT but not significantly. The evidence of optimised healing seen experimentally is supported clinically where a reduction in re-injury rate was found [70].

To date, initial reports describing long-term results of stem cell treatment of tendinous lesions in horses as clinical patients have been published [61, 66]. Results are favourable. Pacini et al. [66] reported a success rate of 90% following MSC treatment of SDFT lesions in 10 race horses, showing that horses successfully returned to their previous level of competition without re-injuring for more than 2 years, while in the non-MSC-treated control group, re-injury occurred in all horses after a median time of 7 months [66]. The biggest clinical trial – with 500 cases of MSC-treated SDFT lesions involved, with long-term follow-up in 82 race horses and in 24 other sports horses – was presented by Smith [61]. Investigating re-injury rates after a 48-week rehabilitation, only 13–36% of the horses re-injured,

including injuries to the contralateral untreated limbs. These results were compared to success rates of 23–66% in all horses after more than 2 years of full work following conventional treatment, published by Dyson [71] and Smith [61]. Own experiences with MSC-treatment of equine tendinous lesions are based on 120 cases, whereof 35% had SDFT lesions and 56% were affected in the SL; success rates for the first group were nearly 80% and over 70% for the second group, these being horses that had returned to their previous level of performance and horses that were in full training [72].

Considering these promising results, it is important to point out that the time of cell injection plays an important role in the success of the treatment. Based on clinical experience, it is suggested that the optimal time for implantation of MSCs is 1–2 months after injury, when a suitable granulation bed has formed and before fibrosis is dominating [68].

Besides the encouraging clinical outcome, the ultrasonographic and post mortem examinations of either clinical [61, 73] or experimental studies [56, 64, 67, 69] also provide promising results. In most cases, ultrasonography revealed that MSC-treated lesions filled in more quickly [73] and showed a linear striated pattern in the longitudinal view [61]. However, Schnabel et al. [67] could not find any significant differences between the treated and their control groups.

Histological findings showed that treated lesions appear to heal excellently and organised collagen fibres in a crimp pattern were found [61, 67] (Fig. 13a, b, c, d).

In the study conducted by Schnabel et al. [67], the effect of Insulin-Like Growth factor I gene transfer to the MSCs was tested additionally, but no significant differences between tendons treated with IGF-I gene enhanced MSCs (AdIGF-MSCs) and unmodified MSCs could be detected. Schnabel et al. [67] also examined mechanical properties, anabolic and catabolic gene expressions, as well as DNA, glycosaminoglycan and total collagen content. Although the treated tendons were stiffer than the controls, and AdIGF-MSC-treated tendons showed an increased gene expression of the catabolic MMP-13, there were no significant differences in all of these parameters. These results suggest that the predominant effect of MSCs on tendon healing is administered through structural organisation.

Another interesting study investigated the possibility of allogeneic MSC application. Autologous and allogeneic GFP labelled MPCs, isolated from bone marrow, were injected separately into SDFT lesions which had been artificially created using a synovial resector blade. At 10 and 34 days after the treatment, no gross and histological qualitative differences between the control lesions and those treated with MPCs could be found in post mortem examinations, which might be due to the short period of time after cell injection. In both cases large aggregations of disorganised cells as well as completely acellular areas within the lesions were detected with haematoxilin and eosin staining. Most labelled cells were located within the MPC-treated lesions, and some were well integrated into the crimp pattern of adjacent healthy tendon areas. A very interesting finding was that no differences in either the number or distribution of autologous and allogeneic cells as well as in the density of leukocytes observed at the respective injection sites were observed, and neither external nor other histological signs of increased

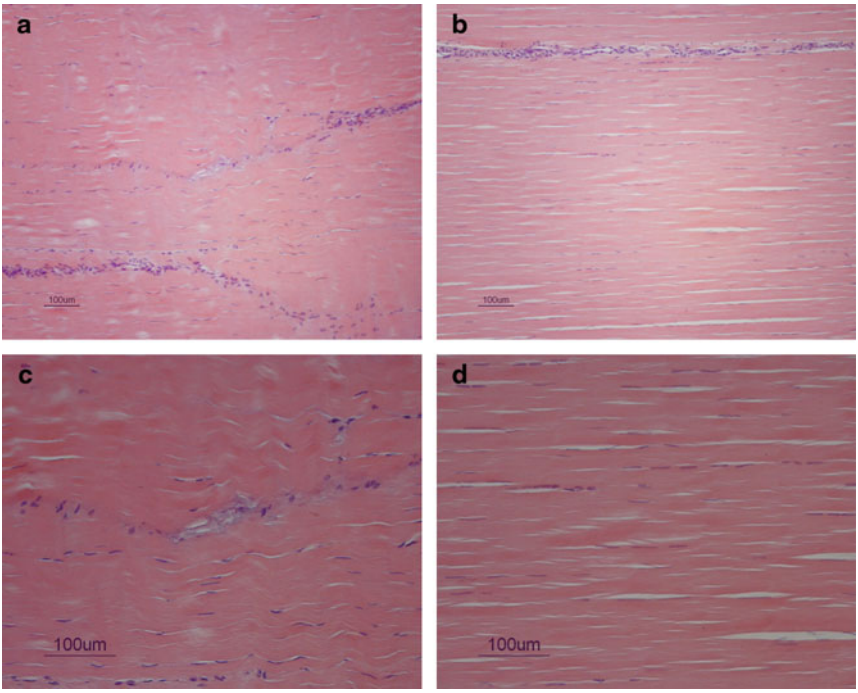

Fig. 13 (a, c) Histology of an MSC treated tendon. (b, d) Histology of a normal tendon

inflammation were found compared to the autologous injection site. This indicates that injection of allogeneic MSCs did not lead to any immune response from the host [56].

In a similar small animal study, autologous and allogeneic bone marrow derived MSCs in a fibrin carrier were implanted into rabbit Achilles tendons. In accordance with Guest et al. [56] it was found that the distribution of inflammatory cells was similar in the allogeneic and the autologous group. No apparent immune reaction such as lymphocyte infiltration associated with the allogeneic transplantation was observed. This seems to confirm that MSCs do not cause alloresponses due to attributed mechanisms such as hypoimmunogenicity and the prevention of normal T-cell responses. Viable allogeneic MSCs were detectable at 8 weeks post implantation. At 3 and 6 weeks following implantation, the cells were shown to migrate around the repair site but in contrast to Guest et al. [56] no migration into the proximal or distal normal tendon was found. Collagen fibres seemed more organised with denser collagen I structures and better biomechanical properties in early tendon healing. At 6 and 12 weeks, however, no differences were detected compared to the group treated with the fibrin carrier alone [57].

Crovace et al. [74] also evaluated the efficacy of local injection of allogenic MSC but in an ovine Achilles tendinitis model. The tendons injected with

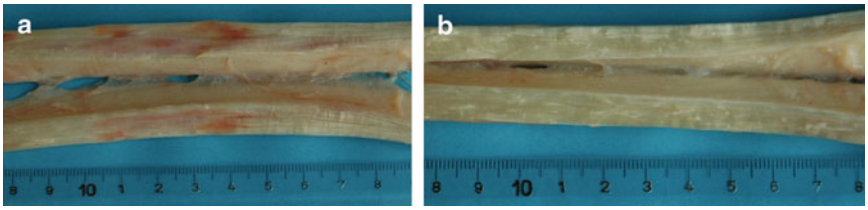

Fig. 14 (**a**) Core lesion in an equine SDFT – typical haemorrhage. (**b**) Normal equine SDFT

allogeneic red fluorescent protein labelled stem cells in fibrin glue showed better architecture of collagen fibres and higher expression of Collagen I compared to control tendons. Moreover, no red fluorescent protein labelled cells were detected in control tendons [74].

As in the studies mentioned above, current investigations of tissue engineered tendons are based on histological and mechanical properties (Fig. 14a, b), due to a lack of specific markers that characterise tendon fibroblasts. A recent study is now analysing a panel of marker genes, which are, in combination, characteristic for adult tendon tissue. Suggested markers are COL1A2, scleraxis and tenascin-C, whereof low expression of tenascin-C and high expression of the former ones distinguish tendon tissue from bone or cartilage [75].

Based on this knowledge, it might be possible to evaluate objectively tendon neogenesis after stem cell application. Further controlled studies will reveal the treatment success with different progenitor cell types, with or without modification, so that optimal tendon tissue promoting MSCs can be identified.

4.2 Osteoarthritis

Osteoarthritis (OA) is a degenerative joint disease with intermittent inflammatory episodes. It is induced by mechanical and biological factors interfering with the normal balance between cartilage synthesis and degradation. These factors, together with inflammatory episodes, lead to softening, fibrillation and degradation of the cartilage surface, as well as to a loss of articular cartilage and sclerosis of the subchondral bone in conjunction with osteophyte formation [146] (Fig. 15a, b, c). The disease can be inherited or induced by one major trauma, several microtraumas or strenuous exercise [76], and leads to pain and decreased range of motion.

OA is the most common human and animal joint disease encountered worldwide. Therefore, MSC therapy for OA is of interest for both human and veterinary medicine and results obtained from research in animals will serve as baseline for clinical trials in humans. Unfortunately, no experimental models that really resemble the pathology of spontaneous OA are available.

However, for single site cartilage defects, several animal models are available.

When choosing a certain species for an OA research study one needs to consider anatomical, physiological and biomechanical aspects as well as availability, handling, ethical concerns and, last but not least, economic aspects.

Rodents are rarely used for cartilage defect models, due to their knee joint size and physiology (growth plates do not close). Rabbits are a useful species for early cartilage defect research; however, important differences in size and physiology minimise their applicability (spontaneous cartilage regeneration in young individuals). Furthermore, dogs can be used for OA research, since they can have defects exclusively involving cartilage tissue (without damaging the subchondral plate), second look arthroscopy can be performed and anatomy and weight bearing is similar to human conditions. But relatively small defect volumes and ethical issues make the dog a less often used species. Small ruminants (sheep and goat) are more commonly employed for preclinical studies as joint anatomy and biomechanical aspects resemble the human situation. A debatable issue is the sheep's variable cartilage thickness (0.4–1.68 mm in different studies) that can produce variable results within the same study. Cartilage thickness seems to be less variable in goats, allowing partial and complete thickness defects. Pigs are a seldom used species for research, due to difficulties with handling and behaviour. The horse represents the largest available animal model and probably the species with most anatomical similarities to humans [77]. Comparable to humans, horses tend to develop spontaneous joint disorders. This is an essential aspect for clinically relevant OA as there might be differences between spontaneous long-lasting and experimentally induced development of OA [78]. Furthermore, cartilage thickness in the equine stifle joint approximates 1.75–2 mm and is therefore comparable to cartilage thickness in the knee of humans, which approximates 2.2 mm. Nevertheless, the differences in human and equine body weights might result in different weight bearings and biomechanical properties within the knee joints [77]. Frisbie et al. [76] compared different animal models with regard to cartilage thickness in the knee joint and observed that the horse is most similar to the human, followed by goat, sheep, dog and finally rabbit.

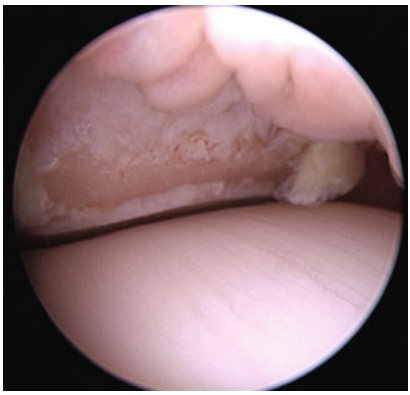

Fig. 15 Extensive cartilage defect (courtesy of Dr. Johannes Edinger)

In veterinary orthopaedics in general, but especially in horses and dogs, joint disease plays a major role. Joint diseases are the most prevalent causes of lameness in horses [79, 80]. Degenerative forms of arthritis constitute approximately one third of all equine lameness, and OA is certainly the most important one [81]. The reasons for the development of OA are not yet fully understood. It is assumed that injury, age and genetics are some of the risk factors [82].

Several epidemiologic studies have shown that lameness due to joint disease is the most significant factor responsible for inability to race and loss of performance in horses [83]. Thus, OA not only has a major impact on equine performance [84, 85], causing morbidity and pain, but is also a major cause of economic loss [86].

Unfortunately, articular cartilage shows only minimal regeneration potential as there is a limited response of cartilage to tissue damage and an inability of natural repair response from adjacent tissues to produce cartilage tissue with morphologic, biochemical and biomechanical properties of healthy articular cartilage. Current treatments include a wide range of non-pharmacological, pharmacological and surgical modalities. Evidence to support the effectiveness of individual treatments, however, is variable [87]. Therefore, the prognosis for patients suffering from OA is still poor. The goals of contemporary management of the OA patient remain control of pain and improvement of joint function as well as of quality of life. However, there are no effective pharmacological therapies available that alter the pathobiologic course of the disease [88]. Therefore, major attempts have been made during recent years to assess the efficacy of regenerative treatments for OA.

For cartilage repair, chondrocytes seem to be the preferred cell type. It is possible to harvest cartilage, isolate the chondrocytes and expand them in vitro. These cells can later be transplanted as fresh or cryopreserved cells. Seddighi et al. [89] found that cartilage engineered with fresh chondrocytes contains more cells and extracellular matrix than constructs engineered with cryopreserved cells. The chondrocytes can be implanted into an existing cartilage defect under a periosteal covering graft [90] or seeded on a collagen membrane which is then transplanted into the cartilage defect [91]. Litzke et al. [92] performed autologous chondrocyte transplantation (ACT) in an equine large animal model. They could show that in comparison to untreated defects, ACT-treated defects had a significantly improved defect filling with well integrated neocartilage.

However, because of the limited cell amount in donors, terminated life span and possible de-differentiation of chondrocytes during the culture period, alternative cell types with chondrogenic potential need to be found.

Currently, adult MSCs are being evaluated for various therapeutic approaches in OA treatment [1, 93].

Well known and practicable sources for MSCs with promising chondrogenic potential are bone marrow [3] and UCB [13, 18]. PB was also used but, in comparison to the other sources, yields were much lower and chondrogenic differentiation was difficult to achieve [15].

It has been shown that MSCs in general – when exposed to TGF ß (Transforming growth factor) – are capable of chondrogenic differentiation and production of collagen type 2 and proteoglycan – two major factors needed for cartilage repair

[1, 3, 4]. Furthermore, Hegewald et al. [94] found that hyaluronic acid and autologous synovial fluid induce chondrogenic differentiation and collagen type 2-production of equine MSCs. Chondrogenic differentiation is also supported by bone morphogenetic protein-4 (BMP-4). In a study conducted by Kuroda et al. [95], muscle derived stem cells, transduced to express BMP-4, were mixed with fibrin glue and implanted into cartilage defects. The results of this treatment showed improvement of cartilage repair up to 24 weeks after transplantation. All these findings suggest that MSCs may be used as a therapeutic agent in OA.

Currently, different techniques exist for the transplantation of MSCs into cartilage defects.

There is the possibility of transplanting differentiated [96] or undifferentiated MSCs, with or without a scaffold. For the implantation without a scaffold, cells can be suspended in various fluids and injected blindly or arthroscopically. Furthermore, they can be fixed with fibrin glue to a certain location. Scaffolds consist of natural or synthetic materials and are usually fixed onto the defect site by suturing, press-fit and/or fibrin glue. MSCs are loaded onto the scaffold either before or immediately after the implantation into the defect and are supposed to expand, differentiate, and produce cartilage matrix.

Nowadays, the intra-articular injection of suspended cells is the most practicable way and therefore most common cell application mode for veterinarians. Agung et al. [1] injected fluorescent-labelled MSCs into rat knee joints with multiple injured tissues (anterior cruciate ligament, medial meniscus and articular cartilage of the femural condyles). Four weeks after injection, they found that MSCs mobilised into some or even all injured tissues depending on the initial number of injected cells. When 1×10^6 MSCs were injected cells migrated only into the injured anterior cruciate ligament (ACL). When 1×10^7 MSCs were injected, cells

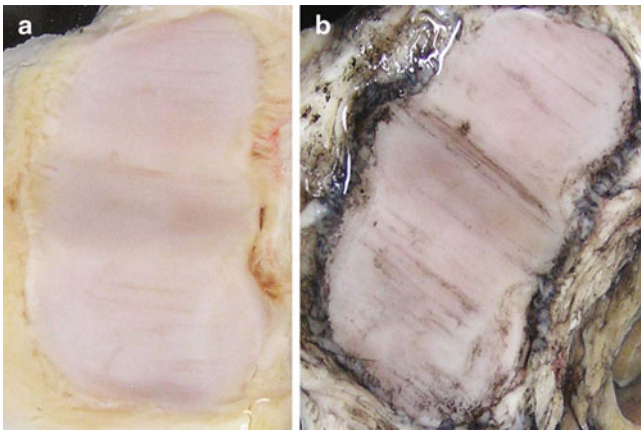

Fig. 16 (**a**) Naturally occurring osteoarthritis – extensive cartilage degeneration with typical wearlines. (**b**) Naturally occurring osteoarthritis – extensive cartilage degeneration with typical wearlines after Indian ink staining

were also found in the injured meniscus and articular cartilage, with extracellular matrix present adjacent to the injected MSCs. However, it was shown that injection of larger numbers of MSCs led to the formation of free scar tissue within the joint, which might have adverse effects on cartilage regeneration. Therefore, determining the optimal number of cells to be injected is essential to minimise problems resulting from unrequested tissues. Regardless of the questions that still need to be solved, Ferris et al. [97] reported about their results of a clinical evaluation of bone marrow derived MSCs in naturally occurring joint disease in horses. Of the 40 horses integrated in the study, 72% returned to work. About half of them returned to or even exceeded their previous level of work. They also found that age, sex, breed and discipline were not significantly associated with outcome. Only the severity of the injury, as classified by the attending veterinarian, was significantly associated with a return to work as four horses who had severe cartilage damage were unable to return to performance. This study confirms anecdotal reports of good clinical outcome post MSC treatment for joint related lesions. Results of this study support future controlled trials to be undertaken for the use of MSCs in horses [97]. Also dogs suffering from OA in their elbow and hip joints were treated with adipose tissue derived cells resulting in an overall clinical improvement of the patients [147, 148]. However, in an equine OA model no difference in lameness improvement between horses treated with MSCs from bone marrow and nucleated cells from adipose tissue [98] was found.

Oshima et al. [99] transplanted undifferentiated green fluorescence protein-marked mesenchymal cells (MCs) rigidly into an osteochondral defect in rats using fibrin glue. It was shown that there were still some marked MSCs in the defect for as long as 24 weeks after transplantation. Also the defects showed better repaired with hyaline-like cartilage than untreated defects.

Wilke et al. [100] implanted undifferentiated MSCs arthroscopically in a self-polymerising autologous fibrin vehicle. The advantage of this technique is the one-step surgical procedure, requiring only one arthroscopy under general anaesthesia. They observed that MSC grafts in horses did improve early healing (1 month) of full thickness cartilage lesions, but the long-term healing (8 months) did not improve compared to untreated defects.

Recently, another new technique of transplanting cells into rabbit cartilage defects, called local adherent technique, was described [34]. Undifferentiated MSCs in suspension were directly placed on the cartilage defect. The defect is pointing upwards and is held stationary for approximately 10 min to allow cell adherence. This easy technique can also be performed via minimal invasive surgery.

Although some studies have provided promising results [1, 93, 101], the efficacy of MSCs in the treatment of OA is still controversial.

To date it is unknown whether tissue regeneration after MSC transplantation originates from the transplanted cells themselves, or whether the transplanted MSCs initiate and support local cells in regenerating the damaged tissue [102]. Another important factor which might contribute to therapeutic success is the anti-inflammatory function assigned to MSCs [103].

4.3 MSCs in Bone Regeneration

The natural repair process of fractured bone occurs via primary and secondary bone union. It is a complex process in which local MSCs generate various essential progenies: chondroblasts, chondrocytes, fibroblasts and osteoblasts forming a fracture callus. Cellular events during regeneration include MSC chemoattraction, migration, proliferation and differentiation into osteoblastic, chondroblasic or fibroblastic lineages depending on the local fracture environment [104]. New extracellular matrix (ECM) is formed and comprises osteoids and cartilage that undergo enchondral ossification and bone formation until the fracture gap is bridged [105].

This natural repair process is efficient for most fractures since the mechanical environment is maintained or created by internal fixation or adjustment. However, specific situations such as tumour resection, trauma, arthrodesis, spinal fusions, metabolic disease or insufficient healing capacities lead to substantial loss of bone. They require augmentation of the natural healing process to regenerate larger quantities of bone. The tissue engineering process of osseous tissue delivers some or all elements required for the natural repair process directly to the site of the large defect. Based on that, three general approaches have been applied to the art of tissue engineering of bone: matrix based therapies that use scaffolding implants to replace the missing bone, factor based therapies that directly provide osteoinductive stimuli such as the family of BMPs and cell based therapies that transfer cells with osteogenic potential directly to the repair site [106]. The latter is based on the implantation of unfractionated fresh bone marrow, culture expanded MSCs, MSCs differentiated towards osteoblastic and chondrogenic lineages or cells that have been modified genetically to express a rhBMP [106]. In general, less differentiated cells are easier to expand in vitro due to their high proliferation rate, while differentiated cells are more effective in vivo due to their higher and rapid production of mineralised ECM.

For both humans and animals, expanded MSCs derived from various tissues (e.g. bone marrow, adipose tissue, periosteum, skeletal muscle) are confirmed to possess osteogenic potential after culture in the presence of dexamethasone, ascorbic acid and glycerophosphate in vitro [15, 18, 107, 108]. Among all adult stem cells, bone marrow-derived stem cells remain the most commonly used cell source for bone regeneration and repair in studies using different animal models [109]. After in vitro findings, the first animal studies were conducted and indicated that MSCs maintain their osteogenic capacity in vivo. Therefore, isolated and expanded MSCs were loaded into porous scaffold matrices and implanted into the subcutaneous tissue of athymic murine hosts where the cells induced the formation of vascularised bone [19]. Next steps comprised the implantation of expanded MSCs and scaffolds into segmental defects in the femur of small animal models (e.g. rats) as shown by Kadiyala and coworkers [19]. By 8 weeks, substantial new bone formation occurred at the interface between the host tissue and the implant, leading to a continuous span of bone across the defect. Furthermore, Richards and

coworkers injected murine MSCs into distracted femoral bones of rats. After 5 weeks they observed significant increase of new bone volume, formation of new trabecular bone with marked osteoblastic activity and osteoid production [110]. These studies established the proof of principle for MSC based tissue regeneration therapy in bone.

So far, the bone regeneration capacity of MSCs to repair various damaged bone tissues such as long bones, cranial bone, mandibular bone and alveolar bone as well as for the enhancement of spinal fusion was examined.

For large segment defects of long bones, Bruder et al. [111] studied the healing of critical-sized osteoperiosteal defects using porous ceramic implants loaded with expanded MSCs. At 16 weeks, radiographic union was established at the interface between the host bone and the implants in samples that had been loaded with MSCs. Significantly more bone was found in the pores of the implants loaded with MSCs than in the cell free implants. In addition, a large bone collar formed around the MSC loaded implants which became integrated and contiguous with callus that formed in the region of the periosteum of the host bone [111]. Other investigators used sheep as alternative species for the segmental bone defect model and confirmed that, after a 2 months period, MSC loaded implants resulted in increased bone formation and accelerated repair compared to unloaded scaffolds [112].

The group of Cui applied adipose derived stem cells and coral scaffolds to repair a cranial bone defect in a canine model. Three-dimensional CT scans after 12 weeks showed that MSC loading of the scaffold resulted in new bone formation while unloaded scaffolds were found partially degraded. Furthermore, radiographic analysis after 24 weeks showed that MSC loaded scaffolds led to more than threefold higher percentages of repair volume than unloaded scaffolds. This study substantiates the potency to apply MSCs and coral scaffold for cranial bone regeneration [113].

In terms of mandibular regeneration, Yuan and coworkers seeded osteogenically induced bone marrow derived MSCs onto a porous beta-TCP scaffold. The cell-scaffold-construct was implanted into critical-sized mandibular bone defects in dogs. New bone formation was observed from 4 weeks after implantation and bony union was achieved after 32 weeks. More importantly, the engineered bone achieved a satisfactory biomechanical property in terms of bending load strength, bending displacement and bending stress [114].

Alveolar bone resorption that is caused by periodontal disease is another field of interest for the application of MSCs in bone regeneration. Weng and coworkers mixed osteogenically induced bone marrow derived MSCs with calcium alginate to create a cell-scaffold-construct in gel form. Those were implanted into alveolar defects in dogs. After 4 weeks bone nodule structures were observed via histology in the tissue. The engineered bone became more mature over 12 weeks, which was similar to normal bone. At 24 weeks the repair level of the alveolus reached nearly half of the height of the normal alveolus showing the applicability of MSCs for alveolar bone regeneration [115].

Next to the described treatments for acute fractures, fracture nonunions and bone defects, MSCs can further be used to achieve therapeutic arthrodesis as necessary

for spinal fusions. Muschler and coworkers developed a rapid, simple and effective method to prepare cellular grafts containing enriched populations of bone marrow-derived MSCs in an implantable matrix of demineralised cortical bone powder. Afterwards, the MSCs enriched cellular graft was implanted into an established canine spinal fusion model. The study showed that a simple aspirate of bone marrow plus demineralised cortical bone powder resulted in an improvement in bone union score, fusion area, and fusion volume compared to matrix alone and matrix with pure bone marrow [116].

Crovace [117] also reported about enhanced bone healing using a resorbable bioceramic based on silicon stabilised tricalcium phosphate and bone marrow mononuclear cells, in a sheep model with a large-sized (4.8 cm), experimentally induced defect in a weight-bearing long bone.

Gardel et al. [118] and McDuffee [119] on the other hand used MSCs which had been differentiated into osteoblasts prior to direct injection into the fracture site in canine patients and a horse model. The former successfully implanted osteoblasts resuspended in PBS into a tibial fracture of a cat. The osteogenic behaviour of the implanted cells was shown by the increased activity of serum ALP after the first and second week of cell application and was in good agreement with the excellent regeneration and bone healing characteristics of the fracture site. Based on the results, MSC application may be considered a possible adjuvant therapy for a quick and successful treatment of long-bone fracture in orthopaedic surgery of small animals but requires further investigation [118].

According to McDuffee [119], periosteal tissue turned out to be the tissue of choice to be used in the in vivo study in a large animal fracture model. Twenty million labelled cells, stimulated to differentiate into osteoprogenitors, combined with a fibrin glue were transplanted into the treatment limb. Fibrin glue alone served as control. Results form five horses demonstrated enhanced bone formation in simulated fractures which received the osteoprogenitor cell-based therapy. Radiographic data showed an increase ($p < 0.05$) in the bone density and histological data a greater percentage of bone area in the limbs which received osteoprogenitor cells compared to control limbs [119].

Another possible indication for the application of MSCs in bone regeneration is Legg–Calvé–Perthés disease in dogs. Legg–Calvé–Perthés syndrome, also known as aseptic necrosis of the femoral head, is a degenerative disease of the hip joint, characterised by loss of bone mass which may lead to a deformity of the femur head and the surface of the hip socket. The disease is characterised by idiopathic avascular osteonecrosis of the capital femoral epiphysis of the femoral head leading to an interruption of the blood supply of the head of the femur close to the hip joint. Small breeds are typically affected. Clinical symptoms are usually seen at a young age (6–8 months). Radiographically the patients show increased opacity and focal lysis in the head of the femur and, later in the disease, collapse and fracture of the neck of the femur. The recommended treatment is surgical removal of the femur head.

Lately Crovace and coworkers reported about the implantation of autologous bone marrow mononuclear cells as a minimal invasive therapy of Legg–Calvé–Perthés

disease in dogs. Prior to implantation the cells were suspended in fibrin glue. Implantation was performed by transcutaneous injection, under CT or radiographic guide, using a Jamshidi needle inserted through the femoral head and neck starting at the base of the trochanter major.

In nine of the treated dogs the disappearance of pain was observed after about 3–4 weeks following cell administration. This also became obvious by a gradual weightbearing on the affected limb up to a complete remission of the symptomatology. In the other two cases a femoral head and neck ostectomy was performed because the recovery proceeded too slowly. Histological and immunohistochemical studies were performed on these samples and showed new formation of cartilage and subchondral bone in the implantation area. Therefore cell therapy seems to be an effective and minimal invasive therapeutic approach for the treatment of Legg–Calvé–Perthés disease. The efficacy is considered to be due to the osteogenetic as well as anti-inflammatory capacity of the stromal cells which may first lead to pain relieve and then to reparative activity within the bone causing a better sclerosis of the femoral head [120].

Regardless of the clinical application, all mentioned studies share the common observation of improved bone tissue formation upon local MSC application as an essential part of the tissue engineering process. However, the application of MSCs for bone repair in the veterinary implementation does not predominantly aim at the clinical treatment of animal patients. More often, animals are used as appropriate models to conduct preclinical studies before advancing to human clinical trials. Still, the principles tested in a species like the dog can directly be clinically translated in the patient of the respective species.

4.4 Spinal Cord Injuries

Acute spinal cord injuries affect many dogs and cats. It has been reported that about 1–2% of all dogs admitted to animal hospitals suffer from injuries to the spinal cord only due to intervertebral disc disease. Clearly there are many other conditions that can lead to compression, concussion or laceration of the spinal cord [121].

Traumatic spinal cord injury causes loss of tissue, including myelinated fibre tracts responsible for carrying descending motor and ascending sensory information. Reduced myelination could be due to either loss of myelinated cells or reduced oligodendrocyte myelin synthesis [122].

Although animals tend to recover a substantial amount of locomotor ability after spinal cord injury, the natural CNS capacity to recover from injury is unfortunately limited. Neuroanatomical differences between species may also be an important factor that needs to be considered in the assessment of the recovery of spinal cord injuries [121].

After spinal cord injury, massive oligodendrocyte death attributed to apoptosis occurs. It seems that a complete restoration of the lost myelin in the injury zone by endogenous oligodendrocytes is not possible. Therefore transplantation of cells

with the ability to differentiate into oligodendrocytes may be a feasible method for myelin replacement. It was reported that stem cells implanted into spinal cord lesions not only differentiate into astrocytes and oligodendrocytes but also integrate into axonal pathways and thus regenerate injured axons [122].

At the moment lots of different sources of cells for neurotransplantation are being evaluated, e.g. embryonic, bone marrow, adipose and UCB stem cells. The cells obtained from these sources can migrate and differentiate into neural phenotypes in the damaged brain and spinal cord [123].

Jeffery et al. [124] showed that recovery of locomotor activity of dogs with spinal cord injuries following autologous olfactory glial cell transplantation appeared to be considerably faster than reported in historical cases.

Adel and Gabr [125] reported significant improvement in the motor power of six dogs compared to the control group, based on intrathecal transplantation of autologous bone marrow derived MSCs 1 week after spinal cord injury.

It was also shown that allogeneic UCB derived MSC transplantation is feasible to induce neuroregeneration using UCB MSCs derived from canine foetuses. UCB contains more mesenchymal progenitor cells and is more pluripotent and genetically flexible than bone marrow derived stem cells. Based on the fact that they are less mature than other adult stem cells they may not elicit alloreactive responses that modulate the immune system.

Dogs included in the study had more than 75% of their spinal canal occluded over a 12-h period. This resulted in a manifest lesion with histologically severe haemorrhage and vacuole formation. The dogs showed paraplegia and were not expected to regain a normal gait. In the group with UCB MSC treatment the gait improved from 2 weeks and the weight bearing of the pelvic limbs improved from 10% to 50% of the time. Therefore, the group with UBC MSC treatment appeared to have improved spinal cord function after the experimentally induced spinal cord injury. It is concluded that MSCs might improve the functional outcome by creating new neuronal pathways in the fibrous scar tissue. They have been observed to integrate into the lesion in the central nervous tissue and a smaller percentage of cavity formation was observed following UCB MSC injection. However, 8 weeks after stem cell implantation magnetic resonance imaging and histology showed no convincing evidence of spinal cord regeneration. Based on somatosensory evoked potentials it was also demonstrated that the nerve conduction velocity was significantly improved. In addition a distinct structural consistency of the nerve cell bodies was observed in lesions treated with MSCs [123].

After human UCB stem cell implantation following spinal cord injury in rats, locomotor function was significantly enhanced within 14 days after transplantation as compared to the non-treated group. In contrast to the non-treated group, consistent plantar stepping, forelimb-hindlimb coordination and no toe drag during walking were observed. Findings demonstrated that hUCB stem cells differentiate into oligodendrocytes and neurons in vivo and lead to improved locomotor function [122]. Moreover, Dasari et al. [122] showed that the number of oligodendrocytes as well as of myelinated axons was elevated in the treatment group compared to the control group and that neuroptropins (NT3 and BDNF) secreted by these

oligodendrocytes in turn enhanced myelinogenesis as well as proliferation and survival of oligodendrocyte precursors. Furthermore, hUCB stem cells producing these neurotropins seem to promote neuritogenesis and axon myelination. Morphologically normal appearing sheaths around the axons in the injured areas were found as well, which was consistent with the observed rapid locomotor improvement. These results are consistent with the hypothesised migration of stem cells to lesion sites and their participation in healing of neurological defects caused by traumatic injury. In the non-injured areas of the spinal cord no hUCB derived cells were detectable [122].

First results from studies in primates (Rhesus monkeys) using bone marrow derived MSCs were promising as well.

Corticosomatosensory evoked potential signals recovered significantly 3 months after MSC injection whereas in control animals the signals remained flattened. The same was observed for motorevoked potential. Healing and regeneration of the spinal cords in animals transplanted with MSC derived cells was shown by H&E staining. In contrast, the injured tissue of the control animals showed obvious degeneration with the appearance of many holes and abundant dissolution of neural tissue and cells. Re-establishment of the axon pathway across the contusive injury of the spinal cord was evaluated by application of labelled cells that were later observed in the rostral thoracic spinal cord, red nucleus and sensory motor cortex [126].

It is not clear, however, whether the therapeutic potential of stem cells is based on their attributed inherent ability to replace injured tissues or if they repair damaged tissue through the induction of neural protection and secretion of neurotrophic factors by various cell types within the graft. More precisely, stem cells could either promote axonal regeneration by constituting a connection through a lesion site which in turn supports axonal attachment or secrete certain growth factors to attract injured axons. It also still needs to be determined if the enhanced functional recovery is based on re-myelination of demyelinated axons or by trophic support to prevent degeneration of the white matter [122].

MSCs have been shown to differentiate into neurons via ex vivo induction as well as following in vivo transplantation. However, compared with native MSCs, neural induced MSCs display a higher survival rate and support better functional recovery after transplantation in rat models. As the microenvironment of acute injury does not favour de novo neurogenesis, the brief induction of MSCs prior to implantation might have a beneficial effect on their in vivo differentiation [126].

4.5 Liver Disease

Also so-called liver progenitor cells (LPC) are hoped to be able to support liver regeneration. LPCs, undifferentiated epithelial cells lying at the interface of the hepatic cords and the biliary tree, offer a promising target for therapeutic intervention in severe liver diseases [127]. They are bipotential cells who express hepatocytic, biliary and progenitor cell markers and can also be isolated from the smallest

and most peripheral branches of the biliary tree (Hering canals) [128, 129]. These cells are defined as side population. Side populations were identified in multiple tissues and display an enriched population of authentic or potential tissue stem cells. In vitro they show a greatly enriched haematopoietic stem cell potential whereas in vivo they show haematopoietic reconstitution activity. In healthy livers, LPCs remain in a quiescent stadium [129] and their presence is low, but they proliferate and invade the liver parenchyma in several pathologic conditions [128]. LPCs are only activated during liver regeneration when hepatocyte proliferation is insufficient [127]. Activated LPCs can either differentiate into haematopoietic lineages [129] or mature hepatocytes as well as cholangiocytes in order to regenerate the pathological changes in the liver [128].

In animal models it was shown that MSCs induced to adopt a hepatocytic phenotype as well as BM mononuclear MSC subpopulations contribute to a histologic decrease in hepatic fibrosis and a rise in serum albumin level when infused early enough after injury onset [130].

The results of the study performed by Arends et al. [129] provide a new option of treatment approach in currently untreatable canine liver diseases. It is hypothesised that liver reconstitution can be stimulated by injection of progenitor cells into diseased livers or via stimulation of the endogenous progenitor cells. The potential use of these cells for the treatment of naturally occurring liver disease in dogs is also of interest for human medicine, as a high homology with human liver diseases at the molecular as well as pathological level is described [128].

Another approach to achieve liver regeneration might be using bone marrow. Bone marrow comprises hepatic stellate cells and myofibroblasts, which were shown to be of MSC origin [131]. Based on these findings it is hypothesised that some hepatocyte regeneration may be achieved through bone marrow MSC transplantation and might induce measurable improvements in hepatic function after damage. Whether engraftment and origin restitution continues in the long term has not been described yet. Another possible explanation for the reduced fibrosis is that hepatocyte proliferation and suppression of fibrogenesis are induced by critical growth factors and cytokines supplied by migrating bone marrow cells [130].

5 Future Prospects and Outlook

Based on all these reports it is obvious that regenerative medicine in the field of veterinary medicine is making great steps to become clinical reality but it is also shown that several important questions still remain to be answered. One of the fundamental questions is the adequate number of cells that would need to be implanted in order to achieve optimal results. Proving that the effect of cell based treatment regimes is in fact caused by the administered stem cells and not by any other cells or biological factors applied simultaneously is still outstanding as well [11]. It also needs to be answered whether stem cells really functionally incorporate into

the tissue that requires regeneration or whether they excite a conducting role recruiting and controlling resident cells to regenerate the respective tissue [11]. Maybe they rather synthesise and secrete growth factors which in turn promote tissue function [5].

Considering all the aspects discussed above, it also seems that MSCs obtained from different sources may have different properties and it will be necessary to define the best source depending on the intended treatment.

6 Embryonic Stem Cells and Induced Pluripotent Stem Cells

Embryonic stem (ES) cells are pluripotent stem cells obtained from the inner cell mass of the blastocyst – an early-stage embryo. In humans, for example, embryos reach the blastocyst stage about 4–5 days post fertilisation. ES cells are capable of self-renewal and thus have the inherent potential for exceptionally prolonged culture (up to 1–2 years). So far ES cells have been recovered and maintained from non-human primate, mouse [5] and horse blastocysts [132]. In addition, bovine ES cells have been grown in primary culture and there are several reports of ES cells derived from mink, rat, rabbit, chicken and pigs [5]. Advances in the laboratory have led to development of feeder and animal-sera – free cells lines. However, clinical application of ES cells remains faced with practical and ethical concerns [133]. Their potential for uncontrolled proliferation and immune rejection [133] as well as their tendency towards teratogenic degeneration in vivo remain major obstacles. The potential to form teratoma consisting of tissues from all three germ lines even serves as a definitive in vivo test for ES cells.

Recently veterinary scientists started to develop several equine ES cells lines so that they can be genetically matched to patients to eliminate immune rejection [133]. Horse ES cells were found to express ES cell marker genes that differ from both human and mouse ES cells, but that reflects the expression of these genes in the inner cell mass of horse blastocysts. Therefore it may be concluded that species differences exist even at this early stage of development and that horse ES cells may provide a better tool to study early horse development than extrapolating data from other species. Equine ES cells are able to generate derivatives of all three germ layers upon differentiation in vitro. Interestingly, they seem not to generate teratomas upon implantation into severe combined immune deficient (SCID) mice. This, combined with a lack of expression of MHC class II antigens, may make horse ES cells more suitable for use in cell transplantation therapies [134] than ES cells from other species. Based on that, at least two companies are currently developing equine ES cells and pilot studies are being performed to determine the efficacy of equine ES cells for tendon regeneration [133]. In addition, ES cells certainly remain an important model system for studying cellular differentiation in relationship to development and oncogenesis [133].

A major breakthrough in the field of stem cell research was achieved in 2006, when it was shown that induced pluripotent stem (IPS) cells could be obtained from

adult somatic cells through expression of a set of transcription factors such as Oct4, Sox2, Klf4, c-Myc, NANOG and Lin28 [133]. Also IPS cells are capable of differentiating into all three embryonic germ layers and, because of this, they have enormous potential for biomedical research and regenerative therapy. These ES-like cells have been generated from rodent, human and porcine somatic cells by forcing the ectopic expression of four transcription factors, Oct4, Sox2, Klf4 and cMyc. These IPS cells may have a great potential in medicine because they can be produced in a patient-specific manner [135]. Generation of IPS cells allows for development of patient-specific cell populations without the ethical controversy of ES cells. Prior to clinical application, an important next step will be to identify ways of assessing which IPS cell lines are sufficiently reprogrammed and safe for therapeutic applications [133]. In addition it will again be necessary to overcome their potential to form teratoma in vivo.

A species, other than humans, that is likely to benefit from this potential is the horse, particularly in regard to the treatment of musculoskeletal injuries. First studies attempting to derive IPS cells from equine somatic cells have begun. Putative equine IPS colonies were identified that tested positive for ALP and Nanog. Clonal populations have continued to expand while maintaining their ES-like morphology over several passages. Current efforts are focused on definitely establishing the pluripotency of these cell lines, including their potential for differentiation into cells of all three embryonic germ layers. Initial results are very encouraging for the eventual generation of IPS cell lines that may have great potential for equine regenerative medicine [135]. However, it was shown that age, origin and cell type have a deep impact on the reprogramming efficiency [136] and most likely also the quality of the obtained IPS cells. IPS cells obtained from somatic cells of adult patients are IPS cells with the biological age of the donor. Therefore, recently a study on the generation of IPS cells from human cord blood was carried out in order to obtain IPS cells from young cells which can be expected to carry minimal somatic mutations and the immunological immaturity of newborn cells [136]. This might offer major advantages for the future use of IPS cells.

In summary, it can clearly be said that in the future a lot of effort still needs to be put into all fields of stem cell research and veterinary medicine will also play an important role because animals may serve as models for human medicine.

7 Animal Models

Cell therapy with adult pluripotent MSCs may revolutionise the treatment of a large variety of diseases in veterinary as well as in human medicine in the future [7]. Veterinary medicine in the form of animal trials plays a major role in preclinical and first clinical phases of human medical trials. In animal studies MSCs seem to provide disease-ameliorating effects in conditions like Alzheimer's disease [137], Huntington's disease [138], amyotrophic lateral sclerosis [139], spinal cord injury [122, 140–142] and myocardial infarction [143, 144].

Also, the effect of MSCs in orthopaedic disorders like OA and tendon injuries is being studied using animal models.

In general, it is important to define the questions and goals of a preclinical animal study before the required species is chosen. However, successful laboratory studies provide valuable proof of principal demonstrating statistical differences in outcome between small groups of treated and control animals with highly uniform injuries, but translational studies aiming to determine whether MSC transplantation provides a medically useful effect in large patient populations that have some variability in the degree of injury severity still need to be carried out [124].

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Bone Marrow Stem Cells in Clinical Application: Harnessing Paracrine Roles and Niche Mechanisms

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Abstract The being of any individual throughout life is a dynamic process relying on the capacity to retain processes of self-renewal and differentiation, both of which are hallmarks of stem cells. Although limited in the adult human organism, regeneration and repair do take place in virtue of the presence of adult stem cells. In the bone marrow, two major populations of stem cells govern the dynamic equilibrium of both hemopoiesis and skeletal homeostasis; the hematopoietic and the mesenchymal stem cells. Recent cell based clinical trials utilizing bone marrow-derived stem cells as therapeutic agents have revealed promising results, while others have failed to display as such. It is therefore imperative to strive to understand the mechanisms by which these cells function in vivo, how their properties can be maintained ex-vivo, and to explore further their recently highlighted immunomodulatory and trophic effects.

Keywords Bone marrow stem cells, Homing and recruitment, Paracrine roles, Regenerative medicine, Stem cell niche

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Abbreviations

BM-MSCs	Bone marrow derived mesenchymal stem cells
BMSCs	Bone marrow derived stem cells
HSCs	Hematopoietic stem cells
MSCs	Mesenchymal stem cells

1 Bone Marrow Stem Cell Based Clinical Trials: Lessons Learned

Innovative in-depth research in the field of stem cell biology has paved the way for the initiation of numerous clinical trials using bone marrow stem cells as therapeutic agents, many of which are already in phases II and III. Currently more than 1,280 clinical trials involving the use of bone marrow stem cells are listed under service of the US National Institutes of Health alone (www.clinicaltrials.gov). Clinical studies published in the past couple of years showed promising results and offered hope for major leaps, particularly in the treatment of cardiovascular disease [1, 2]. However, it is difficult to extract information from many of these trials due to the small number of cases and varying benefits to the patients [3–5]. Perhaps the most limiting factors seem to be the lack of protocol standardization and the shortage of in vivo tracking assays, which make understanding the mechanisms by which these effects are brought about difficult to analyze in human subjects.

1.1 Stem Cell Based Therapies for Cardiovascular Disease

In spite of some inconsistencies, the fact that some beneficial outcomes were noted cannot be ignored. Acute myocardial infarction (AMI) has probably received the most attention of clinically based studies using bone marrow stem cell based therapies [6]. A clinical study conducted on AMI in 60 patients receiving intracoronary injections of autologous mononuclear bone marrow stem cells revealed improvement of both systolic and diastolic left ventricular functions after 6 months.

The injected cells were phenotypically characterized, the majority of those transplanted being CD34+ cells and a smaller fraction CD133+ cells. In contrast to other studies which have shown minimal improvements, the conductors of this study relate positive alleviation of symptoms to several points: the method of introduction of the cells which in this case was intracoronary, the high number of cells used in this study including a major fraction of CD34+ cells, and the elevated initial severity of the condition. However, the number of patients was too low to extrapolate sufficient clinical data [5]. The route of cell-delivery appears as a determining factor in interpreting beneficial results. Preclinical studies indicated that direct intramyocardial cell injection could provide higher efficacy [7] and a recently conducted phase I clinical trial displayed substantial patient improvement up to 12 months post-injection [8]. However, the lack of sham controls and placebos leave unanswered questions, such as to what extent the observed improvement is due to the injected cells.

The time-line over which some of these studies have been conducted also appears to have an effect on the extent of improvements witnessed. While short-term studies have advocated the therapeutic regimen and report significant effects, it has been suggested that multiple injections could be required [9, 10]. Some longer-term studies varying from 18 month to 3 year follow-up of patients who received transplants showed disparate results [2, 4, 11]. However, results of the REPAIR-AMI trial have dissected the processes by which left ventricular remodeling took place within 4 months after injection and have revealed that bone marrow cell injection resulted in earlier remodeling after AMI compared to the placebo group [12]. In these patients, adverse cardiovascular events were significantly reduced and functional improvements persisted for at least 2 years. Most benefits appeared for patients who had presented with a more severe initial status [2].

A meta-analysis conducted over seven controlled clinical studies on patients with AMI revealed that bone marrow stem cell administration significantly enhanced left ventricular functions although two of these studies reported no effect [13]. Although inclusion and exclusion criteria were met, mild variations between these studies did exist, such as differences in cell fraction isolation techniques as well as in the time of bone marrow stem cell administration, number of cells given, methods of evaluation, and follow-up intervals.

Based on recent evidence of the importance of CXCR12/CXCR4 interactions in homing and recruitment, the REGENT clinical trial was conducted using CD34(+) CXCR4(+) bone marrow cells vs an unselected and control group, respectively [3]. After 6 months, the differences in left ventricular function, however, were not significant between the groups, although in patients with more severe illness, results were better in the cell therapy group. In another study, an antagonist of CXCR4 systemically injected in patients with AMI displayed an enhanced capacity of mobilizing CD133+ cells [14]. These effects were observed following a single injection as compared to the need for multiple injections of G-CSF, and did not cause systemic activation of inflammation.

Other approaches have involved the use of precultured allogeneic bone marrow mesenchymal stem cells. A phase I clinical trial using allogeneic BMScs

(prochymal) in patients with AMI has shown a significant increase in ejection fraction that was sustained up to 6 months [1]. The use of more sensitive analytical techniques, such as the cardiac MRI, revealed that some improvements maintained up to 12 months could only be seen in the groups that had received the hBMSCs. Reverse remodeling was also noted in the cell-therapy group, as compared to the placebo group which showed a continuous chamber enlargement; however, these effects were nonsignificant. Although the cells were apparently not retained at the site of damage for prolonged periods of time, the therapeutic effects appeared to be most important in the early post-injury period.

It is highly probable that the paracrine and immunomodulatory effects elicited by these cells could be as important as the exact number of cells engrafted, and whether or not these cells directly contribute to tissue remodeling remains to be seen. A significant improvement of cardiac function has been shown in a rat model of myocardial infarction, whereas the number of cells found in the ischemic heart decreased after 60 min of inoculation and most of the cells were entrapped in the lungs [15]. However, homing was more efficient in ischemic rat hearts as compared to the sham-operated controls. This in fact could be attributed to changes in the local microenvironment and the interaction with the cells which are then capable of activating and recruiting other cells as well as secreting biochemical factors and providing cardioprotective functions. Studies on mice using allogeneic bone marrow stem cells have shown similar detainment times of both syngeneic and allogeneic cells emphasizing the immunomodulatory properties of mesenchymal stem cells and that cell number reduction was not due to immune rejection [16].

Clinical trials using stem cell therapy in AMI have been categorized according to three general approaches; direct injection, indirect cell mobilization using G-CSF, or a combinational approach by first mobilizing the cells and then performing a direct cell injection [6]. In light of the moderate improvements shown from trials using the first and second approaches, the combinational approach has gained favor. This technique may overcome the hurdle of myocardial homing of small numbers of cells by indirect mobilization through the introduction of a successive stem cell injection, thereby enhancing chances of engraftment [17]. A multitude of factors still need to be addressed, such as mechanism of action, lineage of the stem cells and characterization, number of cells, time and method of delivery, homing, follow-up, and imaging of biologic effects [6]. Other applications of stem cell based cardiac therapy are also currently targeting nonischemic dilated cardiomyopathy [18], peripheral arterial disease [19], and ischemic heart failure [20].

1.2 Diligent Candidates for Treatment of a Multitude of Systemic Diseases

Stem cell based clinical trials are also underway for the treatment of a substantially diverse group of systemic diseases ranging from neurodegenerative disorders, stroke, graft-versus-host disease (GVHD), and lately to diabetes mellitus [21].

This is in addition to the use of allogeneic MSCs in the treatment of osteogenesis imperfecta and ankle arthritis [22].

It has been proposed that bone marrow stem cells can be used in the treatment of acute GVHD [23]. Allogeneic mesenchymal stem cells have been shown to enhance engraftment of cotransplanted hematopoietic stem cells in leukemia patients presenting with GVHD as a consequence of a previous MSC injection which resulted in hematopoietic recovery of patients [24]. In a clinical trial involving a group of patients with steroid resistant acute GVHD, half of the patients were found responsive to at least a single injection of bone marrow stem cells. The cells appeared to have a multiorgan effect on reversing GVHD perhaps by suppression of donor T-cell responses to recipient alloantigen [23].

Chronic obstructive pulmonary disease (COPD) and Crohn's disease have also been addressed by stem cell based approaches and are just embarking on the clinical trial phase [25]. Autoimmune diseases such as systemic lupus erythematosus (SLE) may also benefit from allogeneic bone marrow stem cell therapy. SLE has been shown in mice to be associated with osteoblastic niche deficiency which is thereby detrimental to maintenance of the hematopoietic stem cell niche and may contribute to the pathology of SLE [26]. Allogeneic MSCs may act by reestablishing the osteoblastic niche, recovering Foxp3+ cells, and down-regulating Th17 cell levels which are important entities in combating autoimmune diseases. Based on these conclusions, a patient based study was conducted revealing improvements of SLE symptoms upon short-term follow-up [26]. Similar results were displayed in patients with systemic sclerosis receiving autologous hematopoietic stem cell transplantation (HSCT) [27].

The emergence of clinical studies directed towards treatment of neurodegenerative diseases using stem cell based therapy has launched aspiration for many patients. A study on seven patients with Parkinson's disease receiving autologous BM-MSCs has indicated subjective improvements in some symptoms [28]. These are initial results that cannot be generalized at this time but offer immense hope for the future. Other areas include targeting amytrophic lateral sclerosis [29] and stroke [30, 31].

Treatment of diabetes mellitus has lately acquired recognition as a substantial goal of bone marrow stem cell based therapies using different approaches. Type I diabetes mellitus has been shown to be reversible following nonmyeloablative HSCT. The majority of the patients in this study could remain insulin-free for periods as long as 4 years. C-peptide levels were elevated and, even though patients had to resume insulin treatments, this could account for a decreased rate of future complications from diabetes mellitus [32]. Autologous nonmyeloablative HSCT currently appears to be the sole modality capable of reversing insulin dependent diabetes. Consistent positive effects were noted as well in type II diabetic patients [33]. Local cell therapies aiming towards the treatment of chronic diabetic ulcers revealed an improved healing in response to the autologous BM-MSC application in conjunction with wound dressings although biochemical parameters remained unaltered [34].

1.3 From Tissue Engineering to Regenerative Medicine

In the context of tissue engineering approaches, clinical trials utilizing bone marrow derived stem cell/scaffold based strategies are lacking. The bone tissue engineering category, which has been the pioneer field explored, is evidently lagging behind [35]. This can probably be accounted for by several reasons including, but not limited to, the absence of a general consensus on (1) the type of scaffold to be used for distinct bone defects, (2) the type of cell(s) providing optimum bone regeneration in the shortest time, (3) the appropriate biomolecule cocktails, including their time and mode of delivery, the capacity to overcome the revascularization hurdle met with especially in large defects, (4) optimal high-resolution noninvasive method(s) for the evaluation of the implant results, and (5) clinically feasible, safe, and efficient protocol(s) that will result in fully functional dynamic tissues mimicking those of developmental origin.

Long-term follow up of successful reconstruction of challenging long bone defects was documented in four patients who received macroporous bioceramic scaffolds implanted with autologous BM-MSCs [36, 37]. The 6- to 7-year outcome of these patients revealed good integration with complete bone healing and evidence of revascularization in the graft area yet, the scaffolds remained unresorbed. This could later compromise the biomechanical integrity of the regenerated tissue in addition to hindering objective follow-up of the healing process. However, recently a modified silicon stabilized tricalcium phosphate based scaffold has been introduced to address these limitations [38, 39].

Subsequent to these studies, there has been an array of isolated clinical case reports in different application areas. Some aimed to enhance the functionality of allogenic bone grafts by incorporating bone marrow mononuclear cells [40], while others have reported successful long-term results using a combination of bone marrow derived stromal cells and platelet rich plasma as injectable tissue engineered bone for maxillary sinus augmentation [41]. In the latter, alveolar bone height showed consistent increase over 24 months compared to baseline values, although no controls were incorporated in this study.

A similar study showed that bone marrow derived stem cells delivered on a biphasic hydroxyapatite–tricalcium phosphate (HA/TCP) material successfully elevated the sinus compared to initial bone heights and facilitated implant placement in six patients [42]. However in a second study by the same group, little bone formation was seen when BMSCs were combined with a conventional bone substitute in alveolar clefts [43]. It is the recommendation by the investigators that the use of human serum rather than conventional fetal calf serum could have hindered the ability of the BM-MSCs to form bone. Transfer of the cells to the graft substitute as well as the difficulty in establishing bone continuity could be hindering parameters in alveolar cleft regeneration by MSCs based modalities.

A major drawback in these studies is the absence of control patients. Of course, this is not an easy task in a clinical set-up which again reemphasizes the need for controlled clinical trials mimicking the same parameters applied in successful case

reports. Only then can an objective unbiased evaluation be made. Another important setback is the use of heterogeneous populations of stem cells that did not go through in vitro characterization before patient testing [41–43].

A more insightful clinical study investigating the use of autologous BM-MSCs in conjunction with a hydroxyapatite based scaffold for regenerating osseous jaw bone defects prior to dental implant placement documented de novo bone formation in only one of six patients after 4 months [44]. At the same time, a set of synchronized experiments were performed implanting cells from each patient in immunodeficient mice. These revealed ectopic bone formation with cells from all patients;, however, the failure to confirm this clinically was explained by a lack of sufficient vascular supply leading to immediate death of the cells following transplantation. It is hence plausible that bone marrow stem cell–scaffold based strategies need to address the issue of reconstituting a developmentally conductive "niche" which would ensure establishment of a vascular network while maintaining a bed of self-renewing stem cells ensuring dynamic turnover of the tissue [45].

It is apparent that stem cell based clinical therapy is steadfastly gaining momentum yet, until stringent parameters are applied and generalized, a standard clinical application will remain far-fetched. On this basis, more in-depth basic studies have resurfaced, aiming to arrive at more comprehensive explanations for clinical observations.

2 Redefining the Bone Marrow Niche: Implications for Clinical Application

A clinically appealing concept for the use of stem cells is one that allows manipulation of these cells in vivo rather than relying solely on the cumbersome process of ex vivo culture and expansion. These newly founded methodologies would thereby be capable of triggering in-house recruitment and expansion of stem cell populations in a way that would boost the body's own regenerative capacity. This entails a deeper probing of the bone marrow stem cell microenvironment (the bone marrow niche).

A niche is a local microenvironment within which one or more stem cells are housed and maintained. Initially, the niche concept was defined by Grinnell and it was introduced in mammals by Schofield to delineate a microenvironment capable of supporting hematopoiesis [46, 47]. An ideal niche is one that, after a complete elimination of its host stem cell population, could retake a new stem cell and in turn maintain it. Hence, a niche is difficult to replicate in in vitro cultures since these newly introduced environments can alter the patterning of the cells and modify their behavior later in vivo. The existence of facultative niches is a facilitating mechanism to allow homing of stem cells in response to stress or injury. Indeed, signaling profiles of stem cells vary according to the neighboring cells and the physical environment, which further complicates the identification and purification of a purely genuine stem cell [48].

The existence of stem cell niches in the organism is a vital prerequisite to maintaining a constant supply of naïve, undifferentiated stem cells while maintaining lineage development required for the individual's long-term survival. For successful cell based therapy, transplanted stem cells must be capable of homing to appropriate niches, thereby maintaining their lineage development potential and at the same time a constant supply of native cells. In a tissue engineering approach, the ultimate goal would be to engineer an appealing niche within the scaffold, thereby creating a suitable microenvironment for the delivered cells which can be sustained in vivo [49].

2.1 The Bone Marrow Niche: An Orchestra of Cells and Signals

Although stem cell niches should by concept exist in all organs and tissues, little information exists on the nature and mechanism that control these niches. So far, most studies have been concerned with the bone marrow stem cell niche. This is the particular niche within the bone marrow representing a harmonious microenvironment whereby the coexistence of hematopoietic stem cells within their physical microenvironment with bone marrow derived mesenchymal stem cells brings about this balance. In the following section, we will present available information on the bone marrow niche as well as a paradigm of other stem cell niches. Indeed the stem cell niche represents the physical 3D microenvironment within which stem cells are either maintained in a quiescent state, protecting the stem cell reservoir from exhaustion, or under triggering circumstances are prompted to enter the cell cycle and proliferate, mature, or differentiate. From a bone engineering perspective, recreating the stem cell niche is required if a truly hematopoiesis supporting stroma is to be developed within the newly regenerating bone [50–52].

Mesenchymal stem cells have been redefined on a more precise basis as being cells that display plastic adherence, express CD105, CD90, and CD73 in greater than 95% of the culture, and display a lack of expression of markers including CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA-DR in greater than 95% of the culture, in addition to their capacity to differentiate into bone, fat and cartilage [53].

Hematopoietic stem cells are cells capable of self-renewal and giving rise to a cascade of differentiation leading to the creation of all types of blood cells [48]. Hematopoietic stem cells found in adult bone marrow develop from preexisting hematopoietic stem cells that emerged early in ontogenesis, when the bone marrow had not yet formed. In mouse bone marrow, genuine hematopoietic stem cells appear in the bone marrow only after 4–5 days of birth, meaning that they are not responsible for the initial establishment of hematopoiesis but play a major role in its long-term sustenance [54]. During human embryonic development, hematopoiesis sequentially includes the yolk sac, an area surrounding the dorsal aorta termed the aorta–gonad mesonephros (AGM) region, the fetal liver, the bone marrow, and the placenta. However, the properties of hematopoietic

stem cells differ in each of these sites, shining additional evidence on the influential effects of various niches [55].

Within adult bone marrow, hematopoietic stem cells can be more precisely described as groups of cells with varying developmental potentials depending upon signals derived from their cellular niches. It is within this microenvironment that they receive prompting "instructions" either towards blood lineage development or maintenance of self-renewal, i.e., there is a presence of a continuous pool of undifferentiated cells [55].

Identification of hematopoietic stem cells within their niches has been facilitated by the evolution of SLAM family proteins [48, 56]. The identification of SLAM family receptors, including CD150, CD244, and CD48 on the cell surface allowed the definition of the majority of hematopoietic stem cells as related to endothelial cells in vivo [46].

Four possible models of a bone marrow stem cell niche have been depicted: (1) the first relies on adherence of HSCs to perivascular cells and is influenced by nearby endosteal cells; (2) according to the second model stem cells may reside in endosteal niches but can migrate and are subsequently controlled in the perivascular microenvironment by perivascular cells; (3) in the third, stem cells reside in spatially distinct endosteal and perivascular niches; (4) in the last model the stem cells exist in a niche with equal contributions from endosteal and perivascular cells [48].

A positive role of osteoblasts (osteogenic endosteal lining cells) has been depicted using constitutively active PPR (col1-caPPR) under the control of the α1 (I) collagen promoter active in osteoblastic cells in a transgenic mouse. These mice, which had an increased number of trabeculae and trabecular osteoblasts, presented a significantly higher stem-cell-enriched lineage negative (Lin^-) Sca-1^+ c-Kit^+ subpopulation of cells as compared to the wild type animals. This increase was found to be stroma-determined, yet the number of cells in G0 vs G1 was not different between the two types. Furthermore, the PPR activation on the osteoblasts increased the overall production of Jag1 thereby activating Notch signals which led to the expansion of the stem cell fraction [57].

Furthermore, it has been shown that cell-to-cell contact between osteoblasts and hematopoietic stem cells ensures hemopoietic stem cell survival. The physical adjacency of CD34+ bone marrow cells to the osteoblasts triggers the release of several cytokines such as interleukin (IL)-6, leukemia inhibitory factor (LIF), transforming growth factor-β1 (TGF-β1), macrophage inhibitory protein-1α, hepatocyte growth factor (HGF), CXCL12, and IL-7. It has been suggested that quiescent hematopoietic stem cells are maintained by close contact with osteoblasts while their proliferation and differentiation is a function of endothelial cells [46].

Quiescent long-term populating hematopoietic stem cells (LT-HSCs) were found to express Tie 2 tyrosine kinase receptor that interacts with Angiopoietin-1 (Ang)-1 secreted by osteoblasts [58]. They have also been found to be attached to N-cadherin osteoblasts where increased numbers of LT-HSCs were found to be associated with an increase of $CD45^-$ N-$cadherin^+$ osteoblastic cells presenting evidence for the role of N-cadherin in supporting HSCs [59]. However, others have

shown that N-cadherin is not essential for proper hematopoiesis. Through genetic deletion of N-cadherin from HSCs in adult Mx-1^{-} Cre^{+}N-cadherin$^{fl/-}$ mice, no effects on hematopoietic stem cell maintenance were found. It remains possible that the effect of N-cadherin deficiency acts indirectly or that a subset of HSCs do not rely on N-cadherin to localize to the endosteum, and again raises the possibility of perivascular niches [60, 61]. Supportive facts seem to point to hematopoietic stem cell localization to different niches with diverse effects on their properties.

Further evidence for the role of osteoblasts in preserving the quiescent state of HSCs comes from models aiming to devise the osteoblastic niche in vitro. By coculturing osteoblastically differentiated human mesenchymal stem cells with megakaryocytes in the presence of hypoxia, maturation and differentiation of megakaryoctyes into proplatelets was prevented. At the same time, this dynamic interaction led to the deposition of more regularly oriented fibrillar collagen by the human osteoblasts. This in turn led to a feedback inhibitory effect on proplatelet formation mediated through a binding with the integrin $\alpha 2\beta 1$ receptors [62].

In considering the role played by the cells of the osteoblastic cell lineage in the HSC niche, one should notice the major role which is apparently played by stromal preosteoblast cells rather than mature osteoblasts [63]. This sheds light on the rather crucial role that bone marrow mesenchymal stem cells play in regulatory mechanisms of the hematopoietic stem cell niche.

The endochondral ossification route to bone formation also provides additional evidence for modulator functions within the hematopoietic stem cell niche where the formation of a hematopoietic territory appears to take place only via endochondral ossification [16]. Upon ectopic transplantation of mesenchymal stem cells, only CD105^{+}Thy1^{-} mesenchymal stem cells were found to reconstitute both bone and marrow, i.e., they reconstituted a niche generating environment. This was explained by the fact that CD105^{+}Thy1^{-} formed bone through a cartilage intermediate whereas CD105^{+}Thy1^{+} cells did not. Expression of osteoblastic markers was found to be fivefold higher for the latter cells. The mechanism of niche generation was initiated by formation of donor-derived chondrocytes which then recruited host-derived vasculature into the center of the developing graft. As endochondral ossification progressed, hematopoietic centers began to appear first by appearance of erythroid and myeloid, followed by c-kit^{+} progenitors, and finally the HSCs [16].

This evidence also poses questions as to the optimal differentiation route required to optimize bone engineering in a bone marrow mesenchymal stem cell based approach in vivo [64–67]. This is of the utmost clinical relevance and should be used in the future to develop more targeted strategies for tissue engineering, in particular by providing enhanced vascularization. Prepriming of bone marrow mesenchymal stem cells for bone engineering is a rapidly evolving issue for clinical exploitation but it is beyond the scope of this review.

Cross-talk between hematopoietic stem cells and various niche cells has also been demonstrated through other models [62, 68]. Ex vivo real time imaging of stem cells has shown dynamic interaction between HSCs and the bone marrow upon their transplantation in irradiated mice. The HSCs preferentially homed to the endosteal region, yet this preference disappeared in the absence of bone marrow

damage. A mechanism was proposed through expression of SDF-1(CXCL12) which had an increased expression in the trabecular bone area in response to irradiation. In the central marrow zone, vascular signals appear to predominate and the presence of bone marrow damage may give rise to a transient stimulatory environment where osteoblastic signals are reduced and vascular signals are enhanced [60].

The correlation between HSCs and MSCs has likewise been studied. The spatial relationships within the niche through cell-to-cell contacts studied in a three-compartment coculture system of HSCs and MSCs provide insight into their behavioral interconnectivity. Within this system, the cellular localization of HSCs in relation to MSCs affected their expansion. HSCs that had migrated beneath the MSCs retained their stem cell characteristics and proliferated more slowly. β1 integrins and the SDF-1/CXCR4 axis were involved in their migration beneath the feeder layer of MSCs [69]. It has also been shown that contact with MSCs alters the migratory behavior and genetic profiles of CD133+ HSCs ex vivo [70]. Others showed the importance of MSCs in maintaining the hematopoietic environment [71].

Nonetheless, the intricate bond between HSCs and MSCs seems to rely on more than just their physical coexistence. Cotransplantation of HSCs with naïve MSCs alone did not seem to support their self-renewal whereas β-catenin-activated MSCs gave rise to a 4.5-fold increase in the frequency of competitive repopulating units (CRUs) while bone marrow cellularity remained normal. This implies activation of Wnt/β-catenin signals, a concept which may be employed to enhance engraftment of allogeneic transplanted HSCs for patient therapy. It also denotes that successful engraftment may require the preexistence of an activated niche environment [72].

Furthermore, three-dimensional spheroidal culture encompassing noninduced and 1-week osteoblastic induced human bone marrow stromal cells were constructed. In this model, hematopoietic CD34+ cells were seen to migrate freely and lodge to and from the spheroids and could maintain a hematopoietic conductive environment; however, the *osteo*-induced BM-MSCs displayed more strained migration. Specific localization of the CD34+ cells was shown only in mixed spheroids containing both BM-MSCs and osteo-induced BMSCs, showing that both BM-MSCs and active osteoblasts are required for an informative microenvironment. CXCL12 expression increased in the BM-MSCs in the presence of hypoxia [73].

Recent relevance has been given to oxygen levels in the niche microenvironment with discernible proof of the detrimental effects of high oxygen levels on self-renewal of HSCs. Engraftment potential and primitive phenotypes of HSCs appear to be maintained in a hypoxic environment [58]. Slow-cycling HSCs appear to exist in hypoxic zones close to the bone surface and distant from capillaries [74]. SDF-1, which has been shown to be important for HSC homing, is induced by hypoxia inducible factor-1 (HIF-1) and has been found to be abundantly expressed in hypoxic areas of the bone marrow [75].

It is possible that a hypoxic environment functions as a protective mechanism to maintain a pool of quiescent stem cells [58, 62]. This knowledge can again be

tailored to clinical application as many of the diseases showing beneficial effects with bone marrow stem cell therapy share a common phenomenon of oxygen deprivation. Creation of hypoxic zones could provide an effective method of enhancing stem cell recruitment to ischemic tissues and improving repair capabilities. However, the exact oxygen concentration and its duration are not irrefutable and require additional studies, although MSCs cultured in 1% O_2 appear to have reduced proliferation in culture supplemented with platelet lysate over prolonged durations and this appears to be a protective mechanism against DNA damage that may arise with successive replications as well as from free oxygen radical species [76].

Concomitantly shown is the fact that the alterations in the bone marrow microenvironment may be a causative factor in the development of diseases with osteolytic bone lesions such as multiple myeloma. However, repair of these bone lesions, that should operate through mesenchymal stem cells, does not occur in these patients [77]. Similar observations on disease associated changes of the bone marrow niche have been reflected through the altered colony forming efficiency (CFE) of bone marrow stromal cells in a multitude of metabolic, skeletal, and hematological pathologies [78].

As for the long-standing debate as to the location and origin of the bone marrow mesenchymal stem cell and, in turn, the definition of its niche, the European GENOSTEM consortium has recently published an extensive report. They accurately define native bone marrow mesenchymal stem cells to be located on the abluminal side of endothelial cells in sinusoids and that they are the same entity as the stromal cells forming the hematopoietic stem cell niche [79]. It has also been demonstrated by others that mesenchymal stem cells may have originated from the pericytes. Isolated purified pericytes display multipotency and secretion of multiple growth factors similar to those secreted by MSCs. They also express all commonly accepted MSC markers, including CD44, CD73, CD90, and CD105. This could explain the continued presence of progenitor cells with multilineage potential found in virtually all organs. They go further as to illuminate the possibility of the existence of an even more primitive stem cell in human vascular structures [80, 81].

Based on the GENOSTEM experience, they deduce that bone marrow mesenchymal stem cells may in the future be selected using markers of marrow mural/pericyte cells as they have been shown to be multipotential yet preferentially primed to differentiate along mesenchymal and vascular smooth muscle lineages. They also conclude that bona fide stem cells are in fact those that represent clonal highly proliferative culture expandable cells [79].

2.2 Niche Mechanisms and Bases for Stem Cell Homing and Recruitment

Migration, homing, and recruitment of bone marrow stem cells are reliant on their respective niches and their interaction mechanisms. In the previous sections, cell–cell communications within the niche have been discussed with some clarifications

as regards mechanisms controlling these interactions, yet stem cell trafficking is a whole new face of the coin.

It has been shown from a number of clinical trials that bone marrow derived stem cell therapy may provide an efficient means of reconstituting host bone marrow. This may occur through mechanisms involving the recapitulation of signals required to reestablish an appealing host niche, as successful engraftment relies on the availability of open niches with low turnover rates that will support self-renewal, maturation, and differentiation (Fig. 1). Homing is essentially a multi-cascade process that involves intravascular dissemination of stem cells coupled with active migration occurring both before and after the dissemination step. For efficient homing to take place, the cells arriving at the target site must distinguish target-specific signals and enter into a multistep adhesion cascade to adhere to vessel walls in the target organ. Interstitial migration, which is another trafficking mechanism, differs from homing in that it does not require blood flow yet necessitates active ameboid movement of the cells [82].

CXCL12 and angiopoietin-1 expression has been found in endosteal as well as perivascular cells and are thought to be important regulators important for their maintenance [50]. Notch and Wnt signaling have also been suggested although they

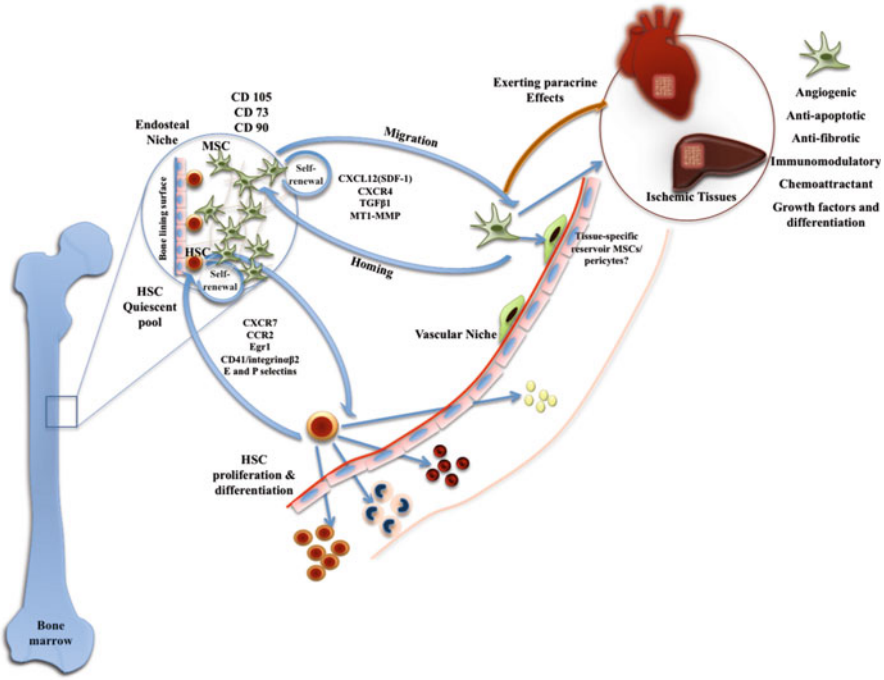

Fig. 1 Schematic diagram depicting some of the numerous stem cell niche interactions and specific roles within played by hematopoietic and mesenchymal stem cells during homeostasis and injury

may not be necessary for adult HSC maintenance in stable conditions but rather upon stress induction. Coordinated processes of symmetric and asymmetric division could also contribute to maintenance of HSCs [48].

A concise review has summed up the major cell-extrinsic factors within the bone marrow microenvironment that are mostly responsible for hematopoietic stem cell regulation. The CXCL12/CXCR4 axis is important for controlling retention of HSCs within the bone marrow as well as the presence of calcium sensing receptors on the surface of HSCs, and a lack of osteopontin may lead to increase in the HSC pool. N-Cadherin appears to play a role although it continues to be controversial and so does the role of Jagged-1 in the activation of Notch1 pathways. On the other hand, maintenance of a quiescent population of HSCs appears to be clearly linked to stem cell factor (SCF), Ang-1, and thrombopoietin [63]. These are in addition to Annexin II, very late antigen-4 (VLA-4)/fibronectin (FN) or vascular cell adhesion molecule-1 (VCAM-1) and leukocyte function associated antigen-1 (LFA-1)/intercellular adhesion molecule-1 (ICAM-1) [46].

Some cell intrinsic factors have also been identified. Profound exploration of intercellular signals reveals molecular mechanisms involved in cellular crosstalk in the bone marrow niche. Upon hematopoietic progenitor and osteoblast cell contact, intercellular transfer takes place. Parts of the hematopoietic progenitor cell membrane are endocytosed at the interface by osteoblasts and delivered to SARA (Smad Anchor for Receptor Activation) – positive signaling endosomes. SARA endosomes specialize in the propagation of extracellular signals such as TGFβ3 and are known to signal through SMAD activation. In response to intercellular transfer, the osteoblasts exhibit greater production of SDF-1 as consequence of a decreased SMAD signaling. This probably occurs because the transferred material in the SARA endosomes sequesters SARA away from its cofactor function in SMAD activation leading to increased SDF-1 production. The cumulative result of these events may influence migration, homing and function of hematopoietic progenitors [83].

Microvesicles (MVs) which are vehicles for mRNA transport have been incriminated in intracellular niche communications as well. They interact with cells through specific receptor ligand interactions leading to direct cell stimulation or by cell surface receptor transfer. Endothelial stem cells (ESCs) are an ample source of MVs and ESC derived MVs can reprogram hematopoietic progenitors by a horizontal transfer of mRNA and protein delivery. The ESC derived MVs can shuttle a specific subset of cellular mRNA such as that associated with eNOS and P13K/AKT pathways [84].

Hematopoietic stem cell homing and migration show a strong involvement of CD41/integrinαb2 during mouse embryogenesis stem cell trafficking. CXCR7 may also be involved. A switch from rapid proliferation to quiescence takes place shortly after HSC homing to bone marrow. The Egr1 transcription factor is the direct molecular link between HSC proliferation and in vivo localization [69, 82]. In addition, CCR2 has been identified as a possible player during hematopoietic stem cell recruitment to the damaged liver in mice, as active recruitment occurred only in wild type mice and not in $CCR2^{-/-}$ mice [85].

For hematopoietic stem cell reengraftment in the host bone marrow, it is likely that preexisting pathways normally used to support HSC physiological circulation to maintain hematopoiesis are also involved to guide efficient engraftment. *In vivo* stem cell homing and migration patterns, however, vary between stem cell lineages and rely to a great extent on how they normally interact with their niches. By understanding these innate migratory mechanisms, stem cells may be exploited as clinical drug or gene delivery vehicles with precise aiming properties [82].

A unique multistep adhesion cascade for HSC homing involves, first, free-flowing HSCs being tethered to the vessel by the vascular selectins, E- and P-selectin, which bind to sialyl-Lewis-like carbohydrate ligands that are associated with PSGL-1 and CD44 on HSCs. Selectin binding, together with engagement of endothelial VCAM-1 with the integrin VLA-4 (a4β1), mediates HSC rolling in marrow sinusoids. The rolling HSCs are then activated by the chemokine CXCL12, which binds to the G protein-coupled receptor, CXCR4. The chemokine signal is thought to induce a rapid conformational change in the VLA-4 heterodimer (VLA-4) that results in increased affinity for VCAM-1 and permits the rolling cells to arrest. Adherent HSCs then migrate into the extravascular bone marrow compartment. Some blood-borne HSCs exit the blood in various peripheral organs where they spend almost 36 h before entering the draining lymphatics. While in peripheral tissues, HSCs can divide and differentiate, presumably to replenish tissue-resident myeloid cells. Through this mechanism, migratory HSCs contribute to immune surveillance by the innate immune system [82].

Hematopoietic stem and progenitor cells (HSPCs) apparently also follow extra-medullary traffic routes shown by the presence of clonogenic HSPCs in mouse thoracic duct lymph, which are capable of short and long-term multilineage reconstitution. HSPCs travel to extramedullary sites where they remain for 2 days before they enter the draining lymphatics and return to the blood. The release of tissue-residing HSPCs into lymphatics seems to occur in response to a lipid S1P gradient which in a similar way regulates the egress of lymphocytes from thymus, spleen, and lymph nodes. This mechanism may serve as part of the innate immune system by which quiescent HPSCs which express TLRs (TLRs recognize foreign molecules such as the bacterial outer membrane component LPS) are forced to enter the cell cycle of myeloid differentiation upon TLR-LPS binding to provide large numbers of cells to boost the number of innate immune effector cells in response to infection or damage [86].

As for migratory mechanisms invoked by mesenchymal stem cells injected in AMI, these maybe confronted with those utilized by leucocytes in response to inflammation. Inflammation-released chemokines trigger an intense release of integrins which propagate firm adhesion to extracellular components followed by their migration from the endothelium through the extracellular matrix (ECM) via the action of ECM degrading matrix metalloproteinases (MMPs). Of these MMPs, MT1-MMP appears to control human MSC collagenolysis and invasion as well as controlling MSC differentiation in 3D in a specific fashion [87]. The adhesion cascade constitutes several steps which start with tethering and rolling, followed by a chemotactic/activating stimulus provided by soluble or surface-bound

chemoattractants, and finally sticking. Both selectin and integrin mediation appear crucial for adhesion [82, 88, 89].

Integrin-mediated adhesion is mandatory if the cells are to cope with shear stresses encountered associated with transendothelial migration. Yet although this maybe the probable mechanism, critical chemokines specifically responsible for MSC migration remain under speculation. It is factual that MSCs have been shown to express various adhesion molecules including CD106 (VCAM-1), CD54 (ICAM-1), CD50 (ICAM-3), CD166 (ALCAM), CD44, and integrins including α1, α2, α3, α4, α5, αv, β1, β3, and β4, many of which are thought to be involved in migration. In particular, high levels of expression of CD44 by MSCs may be directly responsible as blocking CD44 expression markedly reduces the migration of MSCs to damaged kidneys in mice. Signal transduction pathways have gained less attention although Wnt signaling has lately been pinpointed as vital for migration, yet may negatively affect self-renewal properties [88, 89].

In animal models of AMI, myocardial ischemia is found to be responsible for the release of CCL2 (MCP-1), CCL3 (MIP-1α/), CCL4 (MIP-1*β*), CXCL8 (IL-8), CXCL10 (IP-10), and CXCL12 (SDF-1). At the same time, MSCs have been found to express CXCR4 which allow them to migrate in response to CXCL12. However, their expression of CXCR4 appears to be reduced with ex vivo expansion, yet can be enhanced by stimulation with cytokines Flt-3 ligand, SCF, interleukin (IL)-6, HGF, and IL-3. Electin-mediated adhesion has also been suggested to be involved despite the presence of fucosyl transferase in MSCs; an enzyme necessary to generate functional P and E-selectin receptors, remains contradictory in the sense that some researchers have found that MScs have fucosyl transferase (necessary for functional P and E selectin binding) while others (discussed in this reference) have found that they DO NOT have the enzyme and so doubt the involvment of P and E selectin adhesion in MSc migration [88].

High mobility group box 1 (HMGB-1) as well as SDF-1α act as strong chemoattractants for a variety of cell types including stem cells. HMGB-1 is a chemoattractant released during inflammation and cell necrosis and may be involved in recruitment. Furthermore, Rho GTPases which function during adhesion and migration events through actin cytoskeletal regulation have been investigated in trafficking of MSCs. However, neither the Rho nor the Rho effector Rho kinase (ROCK) were found crucial for migration of MSCs in a 3D model. Although others have shown that Rho inhibition induced cytoskeletal reorganization in MSCs, rendering them more susceptible to induction of migration, data remain inconclusive. On the other hand, enhanced migration velocity of MSCs in response to PDGF-B activated fibroblasts points to a positive role of growth factor (bFGF) and epithelial neutrophil activating peptide-78 (ENA-78 or CXCL5) in mediating MSC trafficking. Blocking both bFGF and CXCL5 inhibited both trafficking and differentiation of MSCs while invasion and migration were enhanced when these factors were added exogenously [89].

The SDF-1/CXCR4 axis has repeatedly been shown to play a major role in migration and homing of both mesenchymal and hematopoietic stem cells [82, 90–93]. Hematopoietic stem cells are retained within the bone marrow in a quiescent state by virtue of the SDF-1α/CXCR4 axis, and their mobilization may be

effectively brought about by CXCR4 antagonist coupled with G-CSF treatment, while this same regimen did not effectively mobilize endothelial or stromal progenitor cells. However, when pretreatment with VEGF was administered, hematopoietic stem cell mobilization was suppressed while the mobilization of endothelial progenitor cells was enhanced. Exogenous VEGF stimulated the hematopoietic stem cells to enter into the cell cycle, thereby hindering their migration affinity, whereas it had no effect on endothelial or stromal progenitor cells that retained their ability to migrate in response to administration of CXCR4 antagonist. The effect is only noticed after a few days of pretreatment with VEGF as acute administration did not interfere with endothelial progenitor cell mobilization [91]. Such selective recruitment patterns raise questions as to the therapeutic implications of clinically established protocols and may offer some explanation for some of the disappointing results obtained from some clinical trials.

SDF-1 is up-regulated at sites of bone injury and partakes in endochondral bone repair. Using live and dead bone graft models, SDF-1 expression was shown to be increased markedly in the acute phase of repair in only the live graft periosteum, certifying its role for successful bone healing. Intravenous injection of BMSCs leads to their rapid recruitment to the live graft lesion while blocking CXCR4 using an antagonist inhibited their migration. $CXCR4^{+/-}$ and $SDF\text{-}1^{+/-}$ mice show marked reduction in callus formation and exchanging live grafts between $CXCR4^{+/-}$ and $SDF\text{-}1^{+/-}$ mice revealed that, while live bone grafts from $CXCR4^{+/-}$ into $SDF\text{-}1^{+/-}$ mice could restore decreased bone formation by as much as 52%, the opposite had no such effect [92].

Also worth noting is the migration of osteogenic bone marrow MSCs during the bone remodeling cycle to sites where bone resorption is underway to start a coupling process between bone resorption and formation. Active TGFβ1 is released from the bone matrix in response to osteoclastic bone resorption which induces migration of BMSCs through SMAD family transduction proteins. Depletion of TGF-β1 in mice resulted in massive trabecular bone volume loss, and when these mice received BMSC transplants the cells failed to migrate to the bone surface, showing the essential role of TGF-β1 in inducing migration [94]. From the clinical perspective, the use of bone marrow derived mesenchymal stem cells as a therapy approach for skeletal diseases such as osteoporosis, arthritis, infantile hypo-phosphasia, and osteogenesis imperfecta may be useful, whether these cells are to be exploited by virtue of their capacity to enhance bone repair and regeneration directly or indirectly, or by their spectrum of trophic and immunomodulatory functions [95].

Mechanisms of mobilization and homing/recruitment are vital if stem cells are to be therapeutically used in the clinic. When the mode of delivery is systemic, homing/recruitment mechanisms are the main mechanisms that must be optimized if the cells are to reach the target organs in high enough levels to bring about the desired effect. However, when stem cells are to be utilized as local delivery systems, the need for recruitment signals is surmounted by the need to sustain survival, differentiation, and proliferation if these cells are to contribute directly to local tissue repair. Whether the positive effects brought about by the cells are due to a direct contribution to local tissue repair is uncertain as extensive evidence

seems to point strongly to the paracrine role of injected MSCs as the main reason for the therapeutic effects noticed.

3 Immunomodulation, Trophic Effects, and Angiogenic Supporting Role of Bone Marrow Derived Stem Cells

The trophic effects of mesenchymal stem cells are portrayed by their ability to home to injured tissues and secrete an array of bioactive macromolecules or paracrine factors that are both immunoregulatory as well as regenerative. These capacities lie at the very heart of what is regenerative medicine. It is possible that human MSCs undergoing logarithmic growth are comparable to those that arrive in vivo to sites of injury or ischemia aiming to recuperate lost tissue, since 24 h of MSC culture under osteogenic (+dex, +ascorbate) or stromagenic conditions (+IL-1α) yields conditioned medium with a variety of secreted bioactive molecules such as G-CSF, GM-CSF, M-CSF, LIF, IL-6, IL-11, SCF, IL-3, TGFβ2, and OSM [96].

MSCs have been described to have strong immunosuppressive effects displayed through their ability to inhibit TNFα and INFγ secretion, thus increasing IL-10 secretion, thereby inhibiting T-cell response. This has been supported by the positive effects of allogeneic MSC transplantation to combat GVHD. However, the behavior of MSCs towards the immune system is context sensitive. Low doses of INFγ cause MSCs to express class II major histocompatibility complex (MHC) causing them to behave as antigen presenting cells, while high doses decrease the surface expression of class II MHC and lead to secretion of antiinflammatory factors [96, 97]. The presence of proinflammatory cytokines is necessary to trigger the immunosuppressive function of MSCs [98]. Several reports have noted the need for INFγ to promote the immunosuppressive effects of MSCs; however, others have shown that INFγ alone is insufficient and that the costimulatory activity of either TNFα or IL-1 is required. Nitric oxide (NO) appears to mediate this function. Inducible nitric oxide synthase (iNOS) is upregulated in MSCs stimulated by inflammatory cytokines and the immunosuppressive properties of MSCs are not observed in iNOS deficient mice.

The inherent secretory active nature of MSCs allows them to render a regenerative microenvironment at the site of tissue injury or destruction. This has been duly shown in models of myocardial ischemia whereby the trophic effects of MSCs served to inhibit apoptosis and scarring, stimulated angiogenesis, as well as induced mitosis of tissue residing stem or progenitor cells [96]. Activation of the paracrine pathway of autologous transplanted BMSCs has been achieved in a pig model with acute myocardial ischemia upon the coimplantation of a (bFGF)-incorporated degradable stent (TMDRSI). This method enhanced survival and differentiation of transplanted cells, thereby augmenting their effects on myocardial remodeling [99].

In a similar manner, specific populations of bone marrow derived mesenchymal stem cells have been shown to have superior paracrine effects. Stro-1^+ mesenchymal precursor cells isolated from BM have a tenfold enhanced clonogenic efficiency compared to their plastic adherence selected counterparts. This specific

subpopulation has enhanced paracrine effects as conditioned media from these cells have a much higher capacity to induce endothelial cell migration and endothelial tube formation in vitro as well as superior effects on target cardiac muscle cells [100].

Trophic effects of bone marrow derived mesenchymal stem cells can also be harnessed to promote evolution of noninvasive myocardial stem therapy regimens. Available data from several reports supports the contribution of injected cells, mainly through their paracrine effects [101, 102]. Intramuscular injection of BMSCs and BMSC-conditioned media in a hamster model of heart failure showed that, although the cells appeared to be trapped in the muscle, they were capable of bringing about a multitude of effects that ultimately stimulated active heart regeneration. Myocyte regeneration was evident by the expression of cell cycle markers. Circulating levels of HGF, LIF, and macrophage colony-stimulating factor were associated with the mobilization of c-Kit-positive, CD31-positive, and CD133-positive progenitor cells and with the subsequent increase in myocardial c Kit-positive cells. These should be c-Kit^{+}, CD31^{+}, CD133^{+}. Trophic effects of BMSCs further activated the expression of HGF, IGF-II, and VEGF in the myocardium, emphasizing the precise molecular cross-talks that took place between the injected BMSCs and the host bone marrow compartment and heart. Hopefully, by accurately evaluating effectiveness of this regimen, in the future one may overcome present clinical drawbacks of intramyocardial or intracoronary injection [102].

Such intricate cross-talks can also be displayed in ectopic mouse models of bone regeneration. It has been reported that most of the bone marrow derived sheep mesenchymal stem cells incorporated within ceramic scaffolds disappeared shortly after implantation, and that within 48 h a large percentage of apoptotic cells could be observed [103]. In spite of this fact, bone regeneration still occurred in the ectopic implants as possibly due to the presence of a small number of apoptosis-resistant BMSCs capable of surviving and regaining their proliferative nature.

However, mouse BMSCs implanted in ceramic scaffolds into syngeneic mice gave rise to bone of host origin [104, 105]. Host cells recruited to the implants at 7 days were shown to be a CD31+ enriched population while 11 days after implantation a CD146+ enriched population could be recovered. The latter was dependent on the preoccurrence of the first wave of recruited endothelial progenitor rich population. It was proposed that the exogenous BMSCs release numerous factors in the immediate implant vicinity, thus creating a likable microenvironment to support recruitment of host cells. This fact, combined with newly formed vascular networks, can facilitate the cellular cross-talk between the implant milieu and the host circulation system facilitating the recruitment process. The strong trophic effects noticed in this work with the formation of bone from host origin may be attributed to the implantation of less committed MSCs that are capable of boosting the host response to trigger intrinsic repair as opposed to a more osteogenic committed phenotype that would be tempted to initiate a bone formation cascade.

In addition to the previously mentioned effects, MSC conditioned media has also been found to stimulate differentiation processes of certain cell types such as from neural progenitor cells to oligodendricytes. Therefore, the trophic effects of MSCs can be summed up into antiapoptotic, supportive (stimulate mitosis, proliferation, and differentiation), and angiogenic [96, 97].

The antiapoptotic effects of MSCs are a function of increased levels of vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), and HGF all of which enhance endothelial cell growth and survival. Secretion of GM-CSF, bFGF, and TGF-β has also been reported. Hypoxic culture conditions increase the production of these growth factors, hence explaining antiapoptotic roles of MSCs aiming to minimize cell death as hypoxia occurs in the early stages of injury. bFGF and HGF also contribute to the anti-scaring effects of MSCs by reducing fibrosis, while VEGF, bFGF, placental growth factor (PlGF), and MCP-1 secretion enhance angiogenesis. The secretion of SDF-1 by MSCs aims to retain a pool of quiescent hematopoietic stem cells while the secretion of a larger spectrum of chemoattractant chemokines accounts for the MSC involvement in homing and recruitment mechanisms of a variety of cell types [97].

A major trophic effect of bone marrow derived stem cells deals with their direct and indirect involvement in angiogenesis. Cotransplantation of human bone marrow derived hematopoietic and mesenchymal stem cells in an ectopic model of bone regeneration has been shown to enhance angiogenesis in the implants. The human hematopoietic cells formed stable anastomosis with host vasculature enlightening vascular signaling provided by transplanted cells [52]. When VEGF was added to this model, vessel number and diameter increased; however, bone regeneration capacity was reduced. This is possible as VEGF stimulates endothelial differentiation of CD34+ hematopoietic stem cells which have been previously shown to contribute themselves to osteocalcin expression and engraft at fracture healing sites [106].

In a novel 3D coculture system, BM-MSCs were cultured with human umbilical vein endothelial cells (HUVECs). In this model, 3D lawns of fibroblasts (multilayers) were first created, then on top of these lawns HUVECs were seeded in conjunction with BMSCs. The incorporation of BM-MSCs along with HUVECs led to development and stabilization of tube-like vascular structures in vitro. Furthermore, a subset of the BMSCs was closely coaligned with the newly formed vascular structures [107]. In parallel studies, coculturing BM-MSCs with endothelial cells elicited a time- and dose-dependent increase in vessel formation. The number of sable vessels formed increased when higher proportions of BM-MSCs were used and with longer culture times [108]. As strategies aiming to enhance angiogenesis are at the core of regenerative medicine, discovering the active contribution of bone marrow derived stem cells to angiogenesis is of major interest in the field.

4 Concluding Remarks

The various effects of bone marrow stem cells make them likely candidates for the leap into clinical regenerative medicine and tissue engineering (Fig. 2). The applications are innumerable and will be surpassed only by the further discovery of additional roles for BMSCs in development, disease, and tissue repair [109].

To arrive at defined clinical applications of bone marrow stem cell therapy, meticulous reappraisal appears necessary and the lack of wider scale standardized

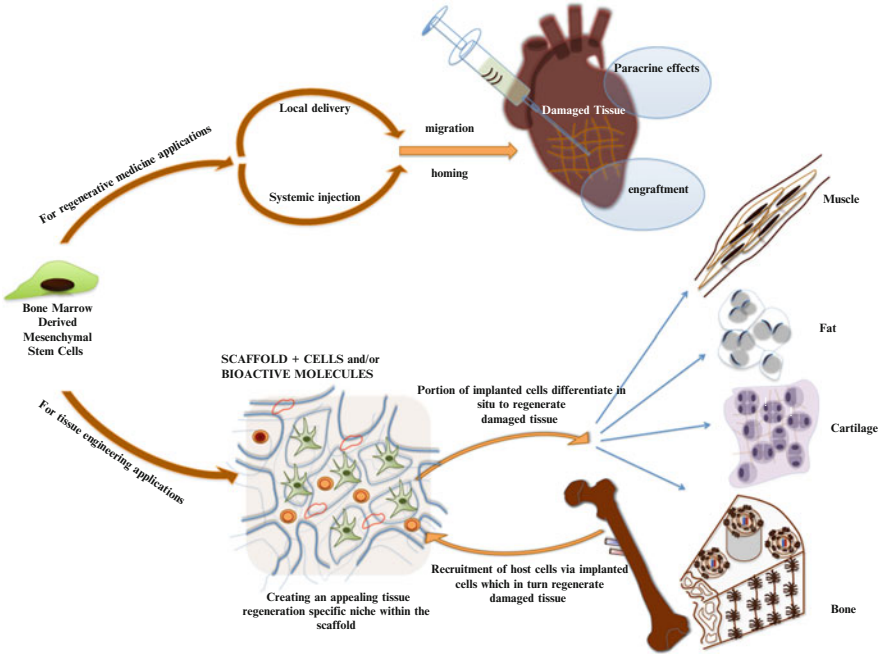

Fig. 2 Possible roles of bone marrow derived mesenchymal stem cells in regenerative medicine vs tissue engineering applications

clinical trials makes it a difficult task. The exact mechanisms by which these cells function in vivo after transplantation remain highly enigmatic. Data from clinical trials show that there are undoubted benefits and the safety of the procedures has been well established, yet, a more profound understanding of the multidimensional aspects of bone marrow stem cell homing, recruitment, and engraftment in vivo is mandatory. More stringent parameters need to be applied to isolate, expand, and characterize these cells if these methods are to move forward as established therapeutic regimens suitable for clinical use. Regenerative medicine has evolved as a growing field aiming to harness the body's innate capacity for regeneration; yet, the comprehensive understanding of these processes still requires delving deep into the basic blocks of developmental biology.

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Clinical Application of Stem Cells in the Cardiovascular System

Christof Stamm, Kristin Klose, and Yeong-Hoon Choi

Abstract Regenerative medicine encompasses "tissue engineering" – the in vitro fabrication of tissues and/or organs using scaffold material and viable cells – and "cell therapy" – the transplantation or manipulation of cells in diseased tissue in vivo. In the cardiovascular system, tissue engineering strategies are being pursued for the development of viable replacement blood vessels, heart valves, patch material, cardiac pacemakers and contractile myocardium. Anecdotal clinical applications of such vessels, valves and patches have been described, but information on systematic studies of the performance of such implants is not available, yet. Cell therapy for cardiovascular regeneration, however, has been performed in large series of patients, and numerous clinical studies have produced sometimes conflicting results. The purpose of this chapter is to summarize the clinical experience with cell therapy for diseases of the cardiovascular system, and to analyse possible factors that may influence its outcome.

Keywords Cell therapy, Heart, Regeneration, Stem cells

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1 Clinical Background

Heart failure is not a uniform disease but has a variety of causes. Quantitatively, the most important is clearly ischaemic heart disease, i.e. coronary artery disease. Occlusion of a coronary vessel results in irreversible necrosis of the downstream myocardium unless the vessel can be reopened within a few hours. Tissue remodelling processes later lead to fibrotic scar formation in the infarct area, but can also affect neighbouring areas of still viable myocardium and may further impair contractile function of the heart. The other causes of heart failure are usually grouped together as "non-ischaemic" and include genetic predisposition, inflammatory heart disease (viral myocarditis, Chagas disease), toxic myocardial damage (doxorubicin, alcohol, cocaine) and structural defects such as valvar disease or congenital abnormalities. Often, however, the underlying cause cannot be clearly established, and the term "idiopathic dilated cardiomyopathy" is used. If the onset

of heart failure is sudden and neither the heart itself nor the other organ systems have had time to adjust to the reduced cardiac output, cardiogenic shock results. The symptoms of this life-threatening situation are mainly the result of a reduced blood flow from the heart (low cardiac output). If heart failure develops more gradually and allows the organism to exert compensatory mechanisms, symptoms are often those of a reduced blood flow to the heart ("congestive heart failure"). According to Neumann et al., the incidence of heart failure requiring hospitalization in Germany exceeds 300 per 100,000 population, and it is currently the most frequent reason for hospital admission [1]. Predominantly older patients are at risk of developing heart failure, and its prevalence among octogenarians is in the range of 10–20%. Heart failure, chronic ischaemic heart disease and acute myocardial infarction are the three most common causes of death, and the costs to the German public health system amount to several billion Euros per year. According to population-based studies, mortality within the first year after diagnosis of heart failure is nearly 40%, followed by an annual death rate of 10% in subsequent years. For several decades, organ transplantation was the only solution for patients with end-stage heart failure but, mainly due to the increasing donor shortage, it is accessible only for a small proportion of patients. In addition, the side-effects of life-long immunosuppression and transplant vasculopathy limit quality of life and long-term graft function. More recently, mechanical assist device therapy is performed not only as a bridge-to-transplantation, but also as the definitive therapy for elderly patients, or patients who have comorbidities that preclude heart transplantation. The design of such assist devices is constantly being improved, but anticoagulation management and infections of power supply lines traversing the skin remain significant problems [2]. Taken together, the current treatment options for the vast majority of patients with heart failure are palliative, because the underlying cause, a net loss of contractile cell mass, cannot be reversed with current therapeutic means.

2 Myocardial Regeneration Concepts

Given the limited efficacy of today's palliative treatment options, the concept of regenerating the diseased myocardium, i.e. improving its functional capacity by restoring the normal tissue composition, is clearly intriguing. For a better understanding of the wide range of "regenerative" therapeutic concepts, a systematic approach may be helpful (Figs. 1 and 2).

2.1 Direct Regeneration

Actual de novo regeneration of contractile cells within the human heart requires the transplantation of cardiomyocytes or their progenitors derived from pluripotent

	Myocardium		
	Indirect regeneration	Direct regeneration	Intrinsic regeneration
Mechanism	Angiogenesis Extracellular matrix Protection of ischemic cardiomyocytes Immunomodulation	Contractile cell replacement Increased number of functional cardiomyocytes	Cardiomyocytes proliferation Activation and differentiation of resident CPC
Therapeutic compounds	Somatic stem cells / progenitor cells Growth factors Laser revascularization etc.	ESC iPS-derived cardiomyocytes	Genetic cell cycle manipulation (Cyclins) CPC activation

Fig. 1 Systematic representation of myocardial regeneration strategies. Examples of the proposed mechanism, its biological mediators, and the respective therapeutic compounds are listed

	Vasculature
Mechanism	Angiogenesis (growth of new blood vessels from pre-existing vessels) • Vasculogenesis (spontaneous blood-vessel formation) and • Intussusception (blood vessel formation by branching of existing vessels) Arteriogenesis (increase in the diameter of existing arterial vessels) Direct suppport of ischemic end-organ cells/tissues Immunomodulation
Therapeutic compounds	Physcial stimuli (hypoxia, hyperoxygenation, heat, ultrasound etc.) Growth factors (gene therapy, protein delivery) Cytokines Somatic stem cells (HSC, MSC, Myoendothelial cells) Endothelial progenitor cells Cells overexpressing growth factors and/or cytokines)

Fig. 2 Regeneration of the vascular system. Here, the systematic approach shown for myocardial regeneration is not applicable. The concepts shown are not only valid for the vasculature of the heart, but also that of any other ischaemic tissue

stem cells. A large amount of experimental data exists for embryonic stem cells (ESC), which can effectively re-generate myocardium but provoke ethical controversies, a risk of teratoma formation and immunologic problems when used in humans [3, 4]. However, the possibility of producing pluripotent stem cells by genetic reprogramming of somatic cells (induced pluripotent stem cells, iPSC) has recently opened up exiting new possibilities [5].

2.2 Intrinsic Cardiac Regeneration

Specific progenitor cells that reside in the heart (i.e. cardiac stem cells, cardiac progenitor cells) may possess a therapeutically exploitable cardiomyogenic potential [6–8]. Alternatively, mature cardiomyocytes, which are terminally differentiated and do not proliferate, might be forced to leave the G_0 resting phase and re-enter the S-phase (DNA synthesis) of the cell cycle [9, 10]. However, DNA synthesis does not necessarily result in genome duplication, and genome duplication does not necessarily result in cytokinesis – division of the cell [9, 11]

2.3 Indirect Regeneration

Somatic human stem cell and progenitor cell products cannot compensate for a loss of contractile tissue in terms of de novo formation of cardiomyocytes [12]. They are, however, able to exert a number of indirect beneficial effects on the diseased myocardium, including the support of angiogenesis and arteriogenesis, modulation of extracellular matrix composition and manipulation of local immune processes [13–16]. Moreover, somatic stem cells seem to be able to protect directly ischaemic cardiomyocytes cell death and/or loss-of-function, but it is unclear whether this phenomenon is clinically relevant [17, 18].

3 The Development of Cardiac Cell Therapy

The goal of cardiovascular regenerative medicine is to restore heart function primarily by biologic means such as the support of intrinsic regeneration processes or the transplantation of exogenous cells. In the 1990s, the concept of cell transplantation for heart failure evolved [19–21]. Initially, myocyte cultures and neonatal rodent cardiomyocytes were used for transplantation in animal hearts [22–25], and the finding that transplanted contractile cells are able to incorporate in postnatal myocardium was ground-breaking. Later, those cells were applied to experimental models of myocardial infarction or non-ischaemic cryolesions, and it was shown that there may indeed be a benefit in terms of contractile function. However, transplanted cardiomyocytes may not be able to survive in terminally ischaemic infarcted tissue [26]. Skeletal muscle progenitor cells (satellite cells, skeletal myoblasts), however, have a very high tolerance to ischemia, the capacity to maintain contractile work even through prolonged periods of anaerobic metabolism, and were shown to form contractile neo-tissue even in scar tissue after myocardial infarction [20, 27]. Indeed, skeletal myoblasts were the first cells to be used for clinical cell therapy, but they were found not to integrate into recipient myocardium and the functional benefit was very limited (see below).

In parallel to the transplantation of contractile cells, marrow-derived cells of haematopoietic–pre-endothelial lineage were shown to be useful for the induction of angiogenesis in the ischaemic heart. The potent pro-angiogenic capacity of marrow-derived cells was initially shown in the mouse hindlimb ischaemia model, and Kocher and colleagues successfully used human CD34+ cells in a rat model of myocardial infarction [13]. Here, the increased growth of small blood vessels in the infarcted heart was associated with a marked improvement of contractility. In large animals, however, the impact of neo-angiogenesis on contractility is less pronounced, while in humans it is often negligible. The most significant – apparent – breakthrough, however, was reported in 2001 when C-kit+ lin− cells were isolated from the bone marrow of GFP expressing transgenic mice and implanted in the infarcted myocardium of non-GFP-expressing animals, and indeed both GFP+ blood vessels and contractile cells were visualized [28]. The conclusion was that adult bone marrow stem cells can differentiate into both endothelial cells and cardiomyocytes, driven by factors present in the surrounding infarcted host myocardium. This report led to clinical pilot studies, in which mainly autologous bone marrow mononuclear cells were delivered to the hearts of patients with myocardial infarction. Other investigators doubted the surprising plasticity of unmodified adult bone marrow stem cells, and reports were published that, using state-of-the-art methods, failed to detect relevant cardiomyocyte differentiation of murine bone marrow stem cells in vivo [29]. Controversy on this issue remains and variations in the technical details of the various experiments are used to help explain the different outcomes. However, even if some haematopoietic stem cells can be driven to express myocyte-specific markers, the frequency of such events in humans is surely too small to produce a significant clinical effect [30]. Mesenchymal stem cells (MSC) from rodents appear to have a greater myogenic potential, provided they are manipulated genetically and/or epigenetically, but only a few studies have provided evidence of functioning cardiomyocytes produced from human MSC in vitro [31–33]. In addition, multipotent cells have been described that seem to belong to none of the typical bone marrow cell compartments but do have a strong regenerative impact on the heart [34]. It is, however, unclear whether such "stemness" characteristics are true features of primary cells or have been induced by long-term cell culture.

4 Clinical Cardiovascular Cell Therapy

4.1 Skeletal Myoblasts

Clinical myoblast transplantation as part of a cardiac surgical procedure was first performed in 2001 [35]. Initial safety and feasibility studies were successful and laid the foundation for the avalanche of cell therapy studies that were to come later [36]. Once a large number of patients had undergone CABG and myoblast

transplantation, however, problems with ventricular arrhythmia were noted. It soon became clear that skeletal myoblasts lack the capacity to couple electrically with surrounding cardiomyocytes because they do not express the intercellular communication protein connexin 43 and thus do not form "connexon" ion channels that are part of the gap junction typical for cardiomyocytes [37]. Several clinical trials using skeletal myoblasts and catheter-based delivery devices are still ongoing [38–40], but the majority of clinicians have abandoned skeletal myoblasts for treatment of heart failure.

4.2 Bone Marrow Mononuclear Cells in Acute Infarction

In theory, no potentially important cell population is missed by transplanting the entirety of nucleated marrow cells into the heart. On the other hand, vast numbers of leukocyte progenitor cells are being delivered in addition to the actual stem cells, and some have argued that more inflammation than regeneration is induced in the myocardium. A clinically very relevant argument in favour of mononuclear cells (MNCs) is their simple and speedy preparation. Traditionally, density gradient centrifugation is used to separate bone marrow mononuclear cells (MNCs) from other marrow components, and industry has developed several easy-to-use devices for one-step preparation of MNC products in closed systems. Bone marrow MNCs were the first cell products to be used in patients with acute myocardial infarction, where they are injected into the infarct vessel that has before been reopened by percutaneous balloon dilation and stent placement (Fig. 3). Following several small-scale pilot trails [41, 42], the first randomized, placebo-controlled study comparing intracoronary MNC injection with standard treatment of acute myocardial infarction was the Hannover BOOST trial [43–45]. At 6 month follow-up, cell-treated patients had a significantly higher left ventricular ejection fraction than control patients. Subsequently, a number of similar studies were conducted by other groups, including a multicenter study that enrolled 200 patients [46–51]. Some of those trials clearly produced a negative result in that there was no difference in outcome between cell-treated and placebo-treated patients [52, 53]. In the multicenter trial coordinated by the Frankfurt group, LVEF rose by 5.5% in cell-treated patients, and by 3.0% in the placebo-treated control group [54]. The difference proved statistically significant, but it remains controversial whether such a small effect will translate into a relevant clinical benefit. Other reports focused on clinical exercise tolerance and quality-of-life data, and again there seems to be a slight advantage for patients who have received cell therapy [55].

4.3 MNC in Chronic Ischaemia

Patients with chronic myocardial ischaemia have also been treated with bone marrow MNC products in several clinical studies. Again, some trials on catheter-based

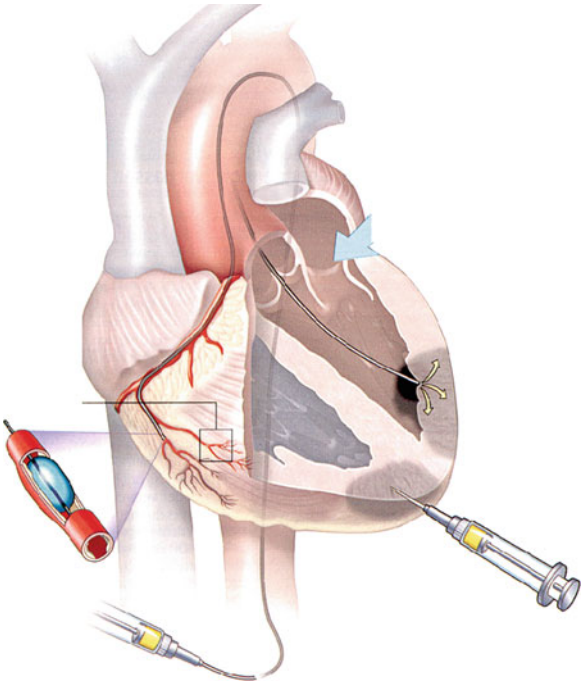

Fig. 3 Delivery of regenerative cell products to the heart. The most frequently used approach has so far been intracoronary injection. Surgical trans-epicardial injection is usually performed together with other cardiac surgical procedures, but stand-alone injection via a mini-thoracotomy is also possible. Catheter-based trans-endocardial injection into the myocardium usually requires complex realtime imaging techniques, and cell delivery is less reliable

delivery of MNCs have shown a modest benefit, while others have produced an essentially negative result [56–58]. The same must be said regarding surgical injection of MNCs in conjunction with bypass surgery. In early pilot studies, an improvement of regional ventricular wall motion in cell-treated areas was observed, but this did not lead to better global heart function as compared with routine bypass surgery [59]. Our own experience was very similar. We treated 14 patients undergoing bypass surgery for chronic ischaemic heart disease and compared their outcome with that of ten patients who had a standard CABG operation. Using a novel echocardiographic analysis tool (ventricular wall strain imaging), we were able to detect improved myocardial function in cell-treated segments (Fig. 4). However, this did not result in better global ventricular function as assessed by LV ejection fraction [60]. In a very recent elegant study, Galinanes and colleagues directly compared intracoronary and intramyocardial injection of MNCs in CABG patients by injecting into the heart muscle or into the bypass graft [61]. Again, they found no relevant benefit of either delivery technique over placebo treatment.

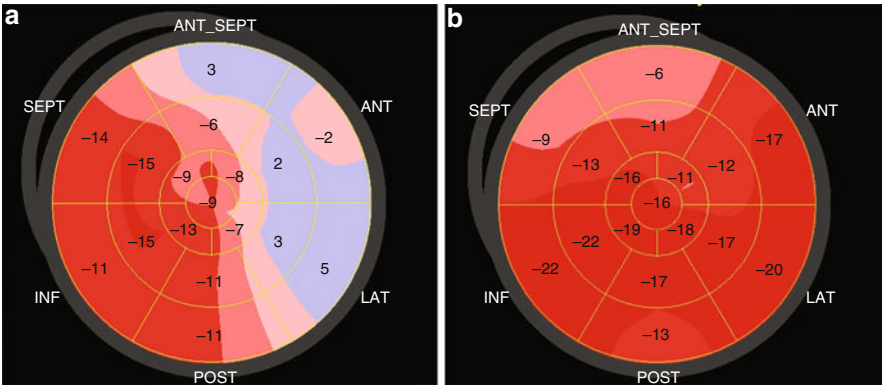

Fig. 4 Two-dimensional longitudinal strain echocardiography analysis of regional left ventricular contractile performance, depicted as "bull's eye view". Preoperatively (**a**) the strain data are inhomogenous, with impaired myocardial motion shown in*blue*. After bypass surgery and bone marrow cell injection (**b**), performance has largely normalized

4.4 MNC in Non-Ischaemic Heart Disease

Initial experimental and clinical cardiac cell therapy studies focussed on ischaemic heart disease, but more recently the potential therapeutic usefulness of cell transplantation for other types of heart failure has been investigated [62–64]. In the recent trial by the Frankfurt group, the effects of selective intracoronary bone marrow cell infusion in 33 patients with non-ischaemic dilated cardiomyopathy were studied. Overall, microvascular function in the coronary system was improved, and the increase of regional contractile function correlated with the functionality of the infused cells as measured by their colony-forming capacity [65]. A decrease in brain natriuretic peptide (NT-proBNP) serum levels also suggested a beneficial effect on left ventricular remodelling processes, and controlled studies are planned to validate these findings.

4.5 Purified Stem Cell Products

Progenitor cell products can be prepared using clinical-grade immunomagnetic selection for either CD34 or CD133, and negative selection for CD45 is also possible. Human bone marrow stem cells of haematopoietic-endothelial lineage, progeny of the primitive haemangioblast, the common precursor of blood and blood vessel-forming cells, can be isolated for clinical use based on the expression of CD34 and CD133 [66, 67]. Theoretically, these cells are potent supporters of angiogenesis processes and hence may be useful for the relief of myocardial ischaemia. Another strategy is the in vitro expansion of bone marrow mononuclear cells, with or without addition of differentiation-inducing or differentiation-suppressing substances. Alternatively,

progenitor cells can be identified based on intracellular enzyme activities, such as aldehyde dehydrogenase (ALDH) activity. ALHD+ cell products from peripheral blood and bone marrow correlate closely with those enriched for CD34 or CD133 but may have superior functional characteristics [68]. Few investigators have used purified haematopoietic stem cell products for the treatment of acute or subacute myocardial infarction. In one such study, concerns were raised about a higher rate of stent occlusion following intracoronary injection of CD133+ cells. Notably, Hofmann et al. studied the cardiac retention of bone marrow cells after intracoronary injection using radioactively labelled cells [69]. When mononuclear marrow cells were used, approximately 2% of the activity was retained in the heart, while the vast majority of the cells accumulated in liver and spleen. However, when CD34 selected cells were used, cardiac cell retention was between 14% and 39%. In chronic ischaemia, however, CD34 or CD133 enriched cells products have found more widespread application [70, 71]. In conjunction with CABG surgery, intramyocardial injection of CD34+ bone marrow cells resulted in nearly 10% higher LV ejection fraction than CABG surgery alone [67]. Our group has focussed on CD133+ cells given during CABG surgery, because they are believed to contain a subpopulation of cells that are even more immature than CD34+ cells. In 2001, we started a feasibility and safety study in ten patients, and no procedure-related adverse events were observed [72, 73]. Subsequently, we conducted a controlled study in 40 patients. Here, CABG and CD133+ cell injection led to a significantly higher LVEF at 6 month follow-up than CABG surgery alone [66]. A pronounced effect on the blood supply to the ischaemic myocardium was evident in numerous patients, indicating the potent angiogenesis support of CD133+ cells (Fig. 5). Other groups have isolated CD133 cells for surgical delivery from peripheral blood, following mobilization from the marrow with G-CSF [74, 75]. This procedure yields a substantially higher cell dose but requires several days for cell preparation. Enriched bone marrow stem cell products (here $CD34^+$ cells) have also been administered by intramuscular injection using catheter-based systems. In a phase I/II pilot trial, this procedure has been shown to be safe, and preliminary data indicate an improvement of LV function over placebo treatment [76].

4.6 Cytokine-Induced Bone Marrow Cell Mobilization

Another strategy aims at circumventing any mechanically invasive procedure for cell delivery and minimizing the interval between the onset of myocardial infarction and cell therapy by mobilizing marrow cells using granulocyte stimulating factor (G-CSF). The idea is that stem/progenitor cells mobilized from marrow will be attracted to the ischaemic heart and initiate regeneration processes [77]. That the number of circulating progenitor cells can be greatly enhanced by G-CSF stimulation has been well established [78]. However, the number of mature leukocytes also rises markedly, and this has raised concerns regarding the safety of C-CSF treatment. In patients with myocardial infarction treated with G-CSF a high rate

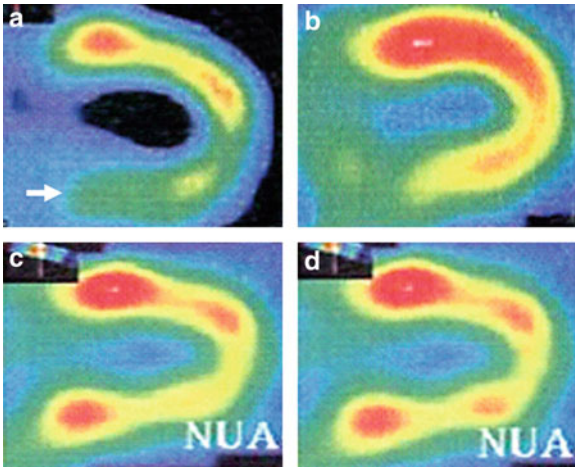

Fig. 5 Perfusion scans of the heart of a patient who had myocardial infarction of the inferior wall of the left ventricle (*arrow*). Preoperatively (**a**), perfusion is several impaired. Two weeks after bypass grafting and transplantation of CD133+ cells in the infarct area, there is no obvious change in blood supply (**b**). Six months later, however, perfusion is effectively restored (**c**), probably via a pro-angiogenic effect of the cell transplantation. This effect is maintained at 1 year follow-up (**d**)

of in-stent restenosis has been observed. Moreover, incidences such as acute re-infarction and sudden death have occurred in other studies [79]. A number of controlled clinical efficacy studies have subsequently been performed, but the outcome data in terms of heart function improvement are rather disappointing.

4.7 Second Generation Clinical Cell Products

Given the modest improvement of heart function after intracardiac delivery of unmodified bone marrow cells, clinical studies are currently being conducted with cell products that have been subjected to in vitro modifications or have been isolated from alternative sources. Among those are mononuclear cells conditioned and expanded in specific bioreactor systems where, theoretically, cells with greater stemness are enriched and their secretory activity is augmented [80, 81]. The frequency of $CD34^+$ or $CD133^+$ cells that are not committed towards a leukocyte phenotype ($CD45^-$) is very low in adult human bone marrow [82, 83], but expansion of haematopoietic stem cells in vitro induces spontaneous differentiation and impairs stem cell function [84]. Bioreactor systems that may be able to suppress stem cell differentiation during proliferation have been developed, but their clinical use in cardiovascular medicine has been limited [85, 86]. The second cellular compartment within the bone marrow, the stroma, contains stem cells that are easier to handle and to manipulate. Multipotent stromal cells, a.k.a. MSC, make

up only 0.01% of the nucleated bone marrow cells but are important supporters of haematopoiesis and act as an independent stem cell pool involved in the maintenance of tissues derived from the embryonic mesenchyme [87]. Moreover, they have been shown to be able to trans-differentiate across lineage boundaries in experimental models, and possess potent immuno-modulatory properties that may also be useful for cardiovascular regenerative medicine [88–91]. Several clinical pilot studies have evaluated the effect of autologous bone marrow MSC in patients with heart disease, but results have been equivocal [92, 93]. Allogeneic donor MSC are also being used in patients with heart disease [94]. They can escape detection and elimination by the immune system, because they express no MHC class II, low levels of MHC class I, and none of the T cell co-stimulatory antigens CD40, CD80 or CD86. Definitive clinical efficacy data, however, are not available yet. MSC from non-marrow tissues are also being evaluated in clinical pilot studies in both autologous and allogeneic fashion. Autologous MSC from adipose tissue can be isolated by plastic adherence in cell culture before they are injected into the heart, while in other trials cells are freshly obtained from a large volume of lipo-aspirate by digestion, washing and centrifugation, and injected intramyocardially without prior cell cultivation [95].

Another interesting development that may help optimize the clinical usefulness of MSC-like cells is the identification of perivascular cells that co-express myogenic and endothelial cell markers and have a robust myocyte differentiation capacity (myoendothelial cells). They are believed to represent the solid-organ reservoir of tissue-specific MSC [96, 97], and have been shown to be very efficient for myocardial regeneration in experimental models.

4.8 Combination Treatments

Numerous strategies to enhance the regenerative capacity of adult stem cells in the heart have been developed. For instance, genetic manipulation of mesenchymal stem cells inducing overexpression of anti-apoptotic proteins such as AKT or Bcl-2, or cytokines such as HGF, IGF or VEGF, not only leads to a marked improvement of MSC survival following transplantation into the heart, but also has beneficial effects on the surrounding host myocardium that comes in contact with secreted anti-apoptotic factors [98, 99]. However, due to patient safety concerns and regulatory restrictions, genetically modified adult stem cells have not yet been tested in the clinical setting. Clinically better applicable strategies to improve cell therapy in the heart are, for instance, hypoxic preconditioning, heat shock treatment or stimulation of cells with cytokines [100, 101], which increase both cellular resilience and paracrine activity. Alternatively, pre-treatment of cells with pharmaceutical agents such as erythropoietin, parathyroid hormone, statins or nitric oxide synthase enhancers has also been applied [102–104]. For both approaches, clinical trials have been initiated. The same is the case for the combination of cell therapy and ultrasound

shock wave treatment [105], which was shown to increase the functional capacity of bone marrow and endothelial progenitor cells [106]. Transplanted cells may survive and function better when they are embedded in an adequate extracellular matrix (ECM) [107], and a clinical trial has been set up where bone marrow cells are mixed with collagen-rich semi-liquid hydrogel prior to implantation in the heart [108]. In this context, the recent development of decellularized cardiac ECM should also be mentioned, which can not only be used as a scaffold for myocardial tissue engineering but will also help understand the interactions between cardiac ECM and transplanted cells [109]. Finally, the combination of transmural laser revascularization (TMLR) and cell therapy deserves to be noted, TMLR is believed to induce a local inflammatory stimulus that supports marrow cells injected into the heart. Again, clinical studies are in progress [110].

4.9 Foetal or Neonatal Stem Cells

The use of juvenile stem cells for cardiac regeneration may solve the problem of age-related and disease-related impairment of stem cell function, and experimental studies have been conducted with cells derived from cord blood, umbilical cord or placenta [5, 111]. However, autologous cells will only be available for patients who had cord blood (or other cells) banked at the time of birth, and large-scale cord blood banking for autologous use has just begun. Some stem cell types from neonatal tissues, however, may have a sufficiently immature immunophenotype to allow allogeneic application [112, 113]. For instance, a clinical trial of allogeneic placenta MSC in patients with severe limb ischaemia is currently going on in our institution [114]. Therapeutic applications for heart disease have not yet been described.

4.10 Paediatric Heart Disease

For children with structural congenital heart disease, tissue engineering may offer new therapeutic options in the future, including the implantation of viable, autologous valves and blood vessels. A number of children, however, suffer from acquired or congenital heart failure without structural defects, and require assist device support and/or heart transplantation. Several cases of cell therapy for myocardial regeneration in children have been reported [115, 116]. Some of these reports were very encouraging, and it has been speculated that the young myocardium may have a greater regenerative potential, and that juvenile stem cells possess greater plasticity and proliferative capacity. Systematic studies, however, have not been performed so far.

5 Clinical Translation Problems

Attention usually focuses on the cell product, but there are many other factors that need to be considered to maximize the likelihood of successful cell-based myocardial regeneration.

5.1 Patient Selection

Every novel therapeutic approach is best evaluated in a uniform cohort of patients with well-defined patient-related and disease-related characteristics. Regarding cell therapy for heart disease, however, this is more difficult than it first seems. In the majority of patients, myocardial cell therapy has been performed within several days after the onset of myocardial infarction symptoms, when patients cannot be selected according to their pre-treatment heart function [43, 47, 53, 54]. Consequently, such patient cohorts cover a wide range of left ventricular contractility at baseline, impeding data analysis and interpretation. In patients with chronic myocardial ischaemia, the degree of contractile dysfunction is usually better defined [66, 67, 74]. Patients with non-ischaemic heart disease have also been subject to cell therapy approaches, although the body of preclinical data is much smaller than for ischaemic heart disease [117]. Here, one problem is the diversity of underlying aetiologies for non-ischaemic heart failure, including inflammatory processes, genetic diseases and a large group of "idiopathic" cases where no cause can be established.

5.2 Cell Delivery

Injection into the coronary arteries requires transmigration of cells through the vascular wall into the myocardium. Catheter-based direct intramyocardial cell delivery is also possible, often combined with intracardiac NOGA mapping of the left ventricle to identify the area of interest (Fig. 3) [56]. A needle-tipped catheter can also be guided into the epicardial veins for injection into the myocardium (reviewed in [118]). For cell delivery under direct vision using surgical techniques, any commercially available syringe and needle system can be used, but industry has also developed special cell injection systems with side-holes. In some trials, cells are being delivered by peripheral venous injection, relying on myocardial chemokines to attract cells to the heart, but the majority undergo first-pass trapping in the lung or are eliminated by the reticuloendothelial system [119]. Each of these cell delivery techniques involves biologic processes that are incompletely understood but probably have a major impact on the therapeutic efficacy.

5.3 Timing

It is not known whether there is an ideal time point for cell therapy in patients with ischaemic heart disease. Emergency treatment of acute infarction is usually done by the interventional cardiologist, who may also decide to perform intracoronary injection of a rapidly available cell product. The situation in chronic ischaemic heart disease is very different. In the post-infarct or chronically ischaemic myocardium, a substantial net loss of contractile tissue mass has occurred and there is diffuse or localized scar formation. Intuitively, the longer the interval between myocardial infarction and cell treatment, the smaller the chance to achieve a beneficial effect becomes. This notion, however, is presently not supported by solid data.

5.4 Cell Survival

When cells are injected into diseased myocardium, most of them do not survive. The magnitude of cell death upon intramyocardial transplantation is difficult to measure, but the suggested survival rate ranges between 0.1 and 10% [26, 120]. The ischaemic heart is a hostile environment, due to local hypoxia, acidosis, lack of substrates and accumulation of metabolites. Moreover, it is infiltrated by phagocytic cells that remove cell debris, and many transplanted cells are probably lost in this "clean-up" process. Third, the mechanic forces in the myocardium are substantial. Transmural pressure is high during systole, there are shear forces between contracting myofibers and layers, and a marrow cell is not well equipped to withstand such stress. However, the rate of cell death can be slowed. Transfection of marrow stromal cells with genes encoding for the anti-apototic proteins AKT or Bcl-2 has been shown to improve greatly cell survival and regenerative capacity [98, 99]. Pre-treatment of endothelial progenitor cells with eNOS-enhancing substances also appears to have a beneficial effect [102], similar to the effects observed with statins [103]. Hypoxic preconditioning or heat shock prior to cell injection might also help, since both activate anti-apoptotic and NO-related signalling pathways [100].

5.5 Dose

The normal adult heart weighs 250–350 g, and around 80% of the myocardial mass consists of approximately 10×10^9 cardiomyocytes [121]. Assuming that 20% of the cardiomyocytes are lost following myocardial infarction, one would ultimately need 2×10^9 surviving neo-myocytes weighing nearly 50 g to reconstitute the myocardium completely. Given the high rate of cell death upon transplantation into the heart and the presumably very low number of adult stem cells that actually differentiate into myocytes, it becomes clear that, with currently available cell products, we are far from being able to replace all lost heart muscle tissue.

5.6 Age

Theoretically, stem cells constantly renew themselves, but it has become clear that bone marrow stem cells undergo ageing processes and are affected by remote diseases [122]. Ageing may not just be imposed on marrow cells by external and internal stressors, but seems to be an active process that helps protect the organism. For instance, upregulation of cell cycle inhibitors of the cip/kip and the INK4a/ARF family reduces the risk of malignancy in the ageing organism. On the other hand, this protective mechanism reduces the stem cell's capacity for self-renewal and proliferation. Indeed, when the cyclin-dependent kinase inhibitor p16INK4a, which accumulates with age, is knocked out in ageing mice, tissue regeneration processes involving haematopoietic stem cells are much improved, but the animals also develop various kinds of malignancies [123]. In essence, the organism sacrifices its regenerative capacity in order to counteract the increasing tendency to develop cancer.

5.7 Disease

It has been well established that ischaemic heart disease is associated with impaired endothelial progenitor cell number and function [124], but it remains unclear whether progenitor cell dysfunction is a cause or an effect of heart disease. On the other hand, exercise has beneficial effects on the somatic stem cell pool. It may be argued that the attempt to repair heart failure with autologous cells that are affected by age and disease is a futile undertaking. The use of allogenic, juvenile stem cells from healthy individuals may be an elegant solution and is propagated by those involved in cell banking activities. However, disease processes in the recipient organism may influence even the behaviour of healthy allogenic cells. Humoral factors in the serum of patients with heart failure have been shown to impair the in vitro proliferation of allogenic bone marrow MSC [125]. Our group has studied the effect of heart failure patient serum on neonatal cord blood mesenchymal stem cells, and we found that, in some patients, MSC proliferation is accelerated, while in others it is severely impaired (Fig. 6). The identification of patients who are likely to benefit from cell therapy and those who will probably not respond is still a largely neglected field of research. Given the biologic complexity of viable cells as therapeutic compounds as well as the organism's response to those clearly requires an individualized approach to maximize the efficacy of cardiac cell therapy

5.8 Legal Framework

Today, the legal situation regarding the production and the therapeutic use of viable cells and related products has been clarified in many countries. Within the EU, this

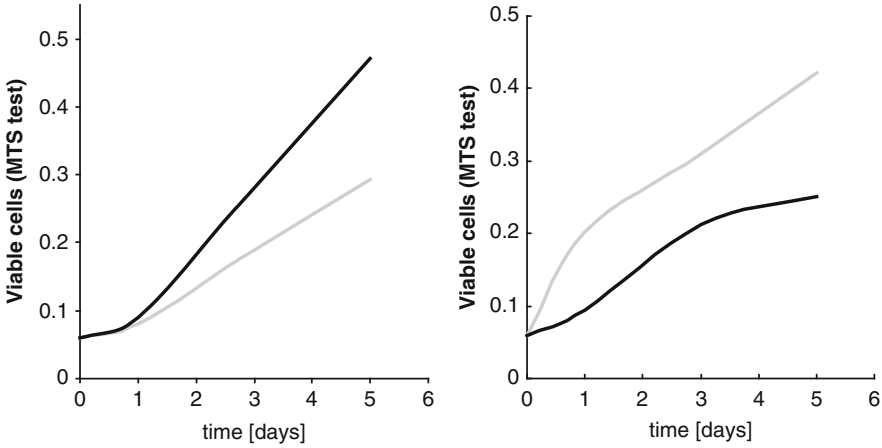

Fig. 6 Serum of patient with heart failure influences stem cell behaviour. (**a**) The in vitro proliferation rate of neonatal cord blood mesenchymal stem cells from a healthy donor, in the presence (*black*) and absence (*grey*) of serum from a patient with ischaemic heart failure. Compared to the standard medium (foetal calf serum), cell proliferation and viability is much reduced. On the other hand, (**b**) shows the in vitro proliferation rate of the same cell type, in the presence of serum from a patient who underwent surgery for ischaemic heart disease (*black*), compared to that of a healthy individual (*grey*). Here, the patient serum clearly has activating effects

was completed with the Regulation on Advanced Therapies No 1394/2007, and the situation is similar in North America. Cells used for tissue regeneration are now considered medicinal products. Hence, their production and clinical application must comply with Good Manufacturing (GMP) and Good Clinical Practice (GCP). Essentially, the same rules are applied that were originally designed to supervise the development in production processing of pharmaceutical compounds. One exception is when autologous cells are harvested and implanted within the same surgical procedure, as is sometimes done in bedside bone marrow or adipose tissue cell transplantation. However, even such "bedside cell therapy products" may be considered medicinal products if the cells undergo more-than-minimal manipulation or their use is non-homologous (meaning the cells are meant to behave in a way that differs from their original function). This regulatory framework has greatly helped optimize the safety of patients who are subjected to still experimental cellular therapies. On the other hand, it has significantly slowed down the clinical translation process.

6 Future Clinical Cell Therapy Studies

Previous and ongoing clinical studies using somatic cells from marrow, adipose tissue and other sources were relatively easy to conduct in accordance with the cell therapy-specific regulations and the standards of evidence-based medicine. The

majority of these approaches were shown to be safe, albeit also with limited efficacy, because they mainly induce indirect regeneration and do not directly interfere with genomic integrity and cardiomyocyte biology. The clinical evaluation of stem cell-derived contractile cells for direct regeneration, however, carries much greater risks and needs a different approach. Because of the tumour and arrhythmia risk when, for instance, iPS cell-derived myocytes are transplanted, we feel that feasibility and safety studies should initially be performed in no-option patients who have or require a mechanical ventricular assist device (VAD). Here, ventricular arrhythmia is usually not life-threatening, and myocardium for histology studies can be collected upon VAD explanation or heart transplantation.

Cell therapy has led to a new level of complexity in both experimental cardiovascular research and clinical practice. The successful use of viable cells for regeneration of diseased tissue requires a completely novel scientific and clinical armamentarium, and it cannot be foreseen whether the efforts will be ultimately rewarded by efficacious and safe regenerative therapies.

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