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Mesenchymal Stem Cells: Basics and Clinical Application I

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Mesenchymal Stem Cells: Basics and Clinical Application I

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Preface

Mesenchymal stem cells (MSC) represent one of the most interesting progenitors to date, due to their biodiverse functionalities. The fascinating multiple properties of MSC are their supportive roles in wound healing and in the regeneration of damaged tissues and organs. This implies the capacity of MSC to migrate toward injured tissue, to undergo differentiation, to modulate the activation of immune cells, and to activate endothelial cells contributing to both angiogenesis and neo-vascularization. Together with their self-renewal capability, the maintenance of stem cell homeostasis, the release of several bioactive compounds like chemokines, cytokines, micro RNAs, and exosomes, MSC can be certainly considered as cellular all-round supporters.

These multi-functional MSC properties are highlighted in the present volume. While some chapters are focused on differentiation capacities of MSC, even beyond the more consolidated mesodermal lineages, others provide novel insights on the stimulatory signals involved in MSC survival and trafficking. Moreover, MSC role in regulating cancer progression for novel therapeutics is assessed. In-depth molecular analyses of MSC functions are also covered; additionally, including initial characterizations of distinct proteomic patterns that are specific for discrete MSC populations. Technical aspects for the isolation and enrichment of selected MSC populations are here additionally addressed in relationship to new cell sources and in the attempt to open new therapeutic platforms for potential clinical applications.

Although MSC research is progressively bridging to more consolidated clinical applications, it still represents a dynamically developing field, where a variety of intriguing aspects remain to be addressed. We feel this volume represents a comprehensive summary gathering a panel of up-to-date articles which combine the diverse MSC biological functionalities and their potential in translational cell therapy, as highlighted from different angles with a broad interdisciplinary perspective.

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Contents

Prospective Isolation and Characterization of Human Bone Marrow-Derived MSCs

A. Harichandan, K. Sivasubramaniyan and H.-J. Bühring

Abstract There is an increasing interest in adult stem cells, especially mesenchymal stem/stromal cells (MSCs), in hematology and regenerative medicine because of the simplicity of isolation and ex vivo expansion of these cells. Conventionally, MSCs are functionally isolated from tissue based on their capacity to adhere to the surface of culture flasks. This isolation procedure is hampered by the unpredictable influence of secreted molecules and interactions with co-cultured hematopoietic and other unrelated cells, as well as by the arbitrarily selected removal time of non-adherent cells prior to the expansion of MSCs. Finally, functionally isolated cells do not provide biological information about the starting population. To circumvent these limitations, several strategies have been developed to facilitate the prospective isolation of MSCs based on the selective expression or absence of surface markers. The isolation and ex vivo expansion of these cells require an adequate quality control of the source and product. Here we summarize the most frequently used markers and introduce new targets for antibody-based isolation and characterization of bone marrow-derived MSCs.

Abbreviations

MSC	Mesenchymal stem/stromal cell
SSEA-4	Stage specific embryonic antigen 4
TNAP	Tissue nonspecific alkaline phosphatase
CFU-F	Colony forming units—fibroblast
PDGF Rβ	Platelet derived growth factor receptor beta

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NK   Natural killer cells
CDCP1   CUB domain-containing protein 1
SSEA-3   Stage specific embryonic antigen 3

Contents

1 Characteristics of Bone Marrow-Derived MSCs

Mesenchymal stem/stromal cells (MSCs) are multipotent cells that are able to form fibroblast-like colonies (CFU-F) [1, 2]. After expansion in culture, bone marrow-derived MSCs express the surface markers CD29, CD73, CD90, CD105, CD106, CD140b, and CD166 but lack CD31, CD45, CD34, CD133, and MHC class II expression [3–6]. They are not only able to differentiate into osteoblasts, adipocytes, and chondroytes, but also into cells of non-mesodermal lineages including hepatocytes, neuron-like cells, and pancreatic-like cells [7–12]. Because of their microenvironment forming ability and multi-lineage differentiation capacity, they present an attractive cell source for co-transplantation with hematopoietic stem cells and replacement therapy for damaged tissues in patients with osteoarthritis, spinal cord injuries, and cardiovascular, neurological, and immunological diseases [13–16].

2 MSCs from Other Tissues

Originally, MSCs were derived from cultured plastic-adherent bone marrow cells. However, a number of other tissues have been identified that contain MSCs at varying frequencies and with varying differentiation capacities. Additional sources with MSC potential include placenta, adipose tissue, peripheral blood, umbilical blood, amniotic fluid, fetal hepatic and pulmonary tissue, skin, and prostate [3, 17–26]. Although cultured MSCs from all sources appear to be negative for CD31, CD45, and CD80, and uniformly express CD9, CD10, CD13, CD29, CD73, CD90, CD105, and CD106, a more tissue-specific expression of other surface antigens has been reported.

For example, only adipose tissue-derived MSCs express high levels of CD34, and only placenta-derived MSCs but not bone marrow-derived MSCs are positive for SSEA-4 and TRA-1-81 [18, 27]. In contrast, bone marrow-derived MSCs but not placenta-derived MSCs express high levels of CD271 and tissue-nonspecific alkaline phosphatase (TNAP) [7, 28–30]. MSCs from different sources not only display differential expression patterns of surface antigens but also vary in their differentiation capacity. It has been demonstrated that bone marrow-derived MSCs display a better chondrogenic differentiation potential compared with MSCs from other sources [31]. Because MSCs are an attractive tool for cartilage tissue repair strategies, bone marrow is considered to be the preferred MSC source for these therapeutic approaches [31].

3 Isolation Procedures of MSCs

3.1 Functional Isolation of MSCs

Conventional procedures to prepare MSCs for research and clinical purposes rely on the expansion of unselected bone marrow cells based on their capacity to adhere to the plastic surface in culture dishes. These functionally isolated MSCs are expanded in defined media in the presence of platelet lysate or other growth factor compositions [4, 32–34]. This isolation procedure is accompanied by several limitations including (i) undesired interactions of MSCs with hematopoietic cells and their released growth factors in the first culture period, (ii) the challenging decision to define the optimal time point of removal of non-adherent cells and replacement with fresh media, and (iii) the co-expansion of other adherent cells (mainly macrophages and endothelial cells) during the expansion period. In addition, functionally isolated MSCs do not provide any information about the antigenic composition of the starting population. As a consequence, most publications describe retrospective antigen expression profiles of MSC progeny but not of the initiating cells. Not surprisingly, a variety of surface markers such as CD109, CD166, and CD318 are exclusively found on cultured MSCs but not on their primary counterparts (Fig. 1) [7, 35]. However, markers such as CD271 or CD56, which are known to be highly expressed on primary MSCs or MSC subsets, are rapidly downregulated in culture (Fig. 1, Table 1) [7]. Markers such as CD13, CD26, CD29, CD73, CD106, and CD140b are expressed both on primary and cultured MSCs (Fig. 1). Despite the limitations of functional isolation protocols, these procedures are still prevalent for large-scale MSC preparations in clinical settings because expensive GMP-manufactured antibodies for immunoselection are not required.

3.2 Prospective Isolation of MSCs

In contrast to functional isolation procedures, the prospective isolation of MSCs allows a precise definition of the starting population. This isolation procedure also precludes the potential adverse effect of co-cultured hematopoietic cells and avoids
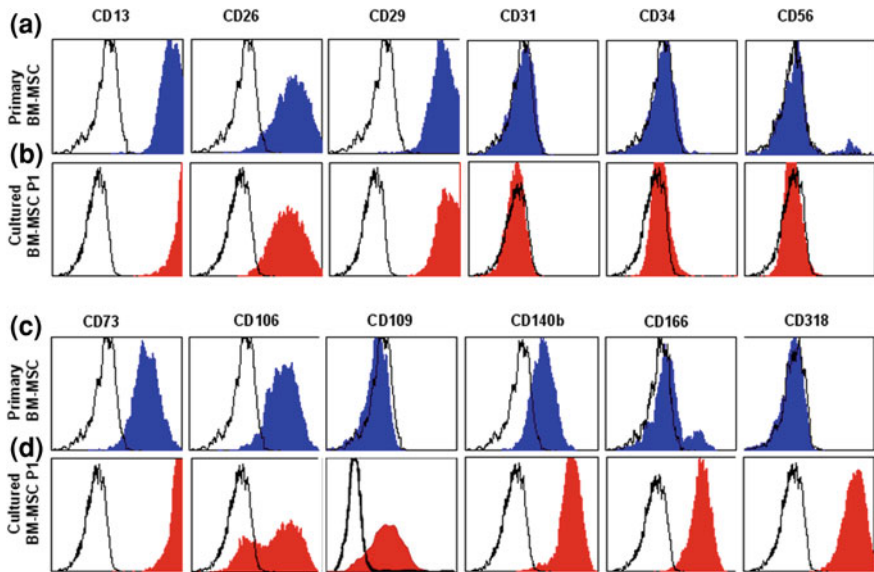
Fig. 1 Comparitive phenotype of primary bone marrow (BM)-derived CD271^bright CD45^− BM cells and cultured MSCs. **a, c** One million BM cells were stained with CD45, CD271, and test antibodies and analyzed by flow cytometry using a FACSCanto II analyzer. After gating on the CD271^bright CD45^−/dim population, the expression of selected CD markers was analyzed. **b, d** Expression of selected markers on cultured MSCs derived from unfractionated BM cells. MSCs from passage 1 (P1) were stained either by direct or indirect immunofluorescence and analyzed on a FACSCanto flow cytometer. *Black lines* represent control staining

the potential removal of important MSC subsets together with other nonadherent cells. In addition, no other adherent cells are co-cultured that may interfere with the expansion of MSCs. To isolate MSCs from primary bone marrow or other tissue, several markers were identified, which are suitable to enrich for these cells. These markers include antibodies against a variety of surface molecules including CD49a, CD63, CD73 (SH3/SH4), CD105 (SH2), CD106, CD140b, CD271, TNAP, and Hsp90-beta, as well as orphan antigens defined by antibodies STRO-1, W3D5, and W5C5 (Table 2a, 2b). In some cases, MSCs were not selected (or not only selected) by their immunophenotype but rather by functional features such as the enzymatic activity of aldehyde dehydrogenase, which is known to be increased in stem cells of many tissues [36]. Using Aldefluor as a specific dye to monitor this enzyme activity, a 9.5-fold enrichment of bone marrow-derived MSCs in the Aldefluor^bright population was achieved (Table 2a) [36]. In other approaches, bone marrow-derived MSCs were enriched by negative selection, employing markers such as CD14, CD34, CD45, and/or CD235 (glycophorin A) and other "lineage-negative" markers (Table 2c).

Distinct markers are required for the selection of MSCs from sources other than bone marrow because of some unique phenotypic peculiarities. For example, placenta-derived MSCs are preferentially isolated using antibodies against CD349

Table 1 Differential expression of surface markers on primary and cultured bone marrow-derived MSCs

Primary MSCs		Cultured MSCs (passage 2)
CD56$^+$	CD56$^-$	
CD10$^-$	*CD10$^+$*	*CD10$^+$*
CD13$^+$	CD13$^+$	CD13$^+$
CD26$^-$	*CD26$^+$*	*CD26$^+$*
CD34$^-$ (subp.$^+$)	CD34$^-$	CD34$^-$ (some clones$^+$)
CD49a$^+$	CD49a$^+$	CD49a$^+$
CD49b$^-$	CD49b$^-$	CD49b$^-$
CD56$^+$	*CD56$^-$*	*CD56$^-$*
CD90$^+$	CD90$^+$	CD90$^+$
CD105^{dim}	CD105^{bright}	CD105$^+$
CD133$^-$	CD133$^-$	CD133$^-$
CD140b$^+$	CD140b$^+$	CD140b$^+$
CD146$^{-/dim}$	*CD146$^+$*	*CD146$^+$*
CD166$^+$	*CD166$^-$*	*CD166$^+$*
CD318$^-$	*CD318$^-$*	*CD318$^+$*
CD271$^+$	*CD271$^+$*	*CD271$^{-/dim}$*
TNAP$^{-/dim}$	*TNAP^{bright}*	*TNAP$^{+/-}$*
SSEA3$^-$	*SSEA3$^+$*	*SSEA3$^-$*
W5C5$^+$	W5C5$^+$	W5C5$^+$
2B1H4$^+$	*2B1H4$^-$*	*2B1H4$^+$*

Markers, which are exclusively expressed on a distinct primary MSC subset or on cultured MSCs, are highlighted in italics

(frizzled-9), SSEA-4, and TRA-1-81, which is in contrast to their bone marrow-derived counterparts that are preferably isolated by CD271 or TNAP selection [18]. Other markers, such as CD34 and CD117, are more suitable to select adipose- and amniotic fluid-derived MSCs, respectively (Table 2a) [37].

The CFU-F assay is the most frequently used test to analyze the clonogenic potential of prospectively isolated MSCs [1, 2]. Candidate antibodies selective for MSCs can be evaluated by screening their reactivity with cell populations that express established key MSC markers, such as CD271 or STRO-1. An example is shown in Fig. 2, in which bone marrow cells are double stained with antibodies against CD271 and CD140b (PDGF receptor-beta). The FACS plot demonstrates that only CD271^{bright} but not CD271^{dim} cells give rise to clonogenic MSCs, and these populations differ considerably in their morphological appearance. Giemsa staining shows that CD271^{bright} cells are characterized by a relatively bright nuclear staining and a high cytoplasmic content, compared to the immature lymphoblastoid appearance with darker nuclear staining of CD271^{dim} cells. The plot additionally shows that CD140b is a more selective marker for MSC isolation than CD271 because this molecule is expressed only on CD271^{bright} but not CD271^{dim} cells. As expected, clonogenic cells (CFU-F) were exclusively found in the CD140b$^+$ population. Using this screening approach, additional antibodies with specificity for MSCs have been identified [7, 18, 28, 29, 38] and may be discovered in the future.

Table 2 List of antigens/antibodies used for the prospective isolation of MSCs

Markers used	Tissue	References
(a) *Known antigens for positive selection*		
CD9 (MRP-1; MIC3)	Synovial membrane	[46]
CD10 (neprilysin; CALLA)	Placenta	[18]
CD26 (DPP4)	Placenta	[18]
CD34 (MY10; gp105–120)	Adipose tissue	[23–25, 47–49
CD44 (PGP-1; ECMR-3)	Bone marrow	[50]
CD49a (integrin α1)	Bone marrow	[51–55]
CD49e (integrin α5)	Bone marrow	[56]
CD56 (NCAM)	Bone marrow	[7, 28, 29]
CD63 (MLA1; TSPAN30)	Bone marrow	[54]
CD73 (NT5E)	Bone marrow	[57–59]
CD90 (Thy-1)	Adipose tissue	[24, 25]
	Bone marrow	[60]
	Synovial membrane	[46]
	Endometrium	[61]
CD105 (endoglin)	Synovial membrane	[62]
		[50, 58, 59, 63–65]
	Bone marrow	[66]
	Cartilage	[67]
	Endometrium	[68]
	Wharton's jelly	
CD106 (VCAM-1)	Bone marrow	[69, 70]
	Umbilical cord	[22]
CD117 (C-Kit)	Amniotic fluid	[37]
CD130 (gp130)	Bone marrow	[57]
CD140b (PDGFRB)	Endometrium	[71]
CD146 (MCAM)	Bone marrow	[57, 72]
	Adipose tissue	[17, 73]
	Endometrium	[61, 67, 71]
CD166 (ALCAM)	Synovial membrane	[46]
	Cartilage	[66]
	Bone marrow	[54]
	Fetal membranes	[3]
CD200 (MRC, OX2)	Bone marrow	[57]
CD271 (LNGFR)	Amnion	[3]
	Bone marrow	[7, 28, 29, 34, 38, 65, 74–77]
	Chorion	[3]
	Adipose tissue	[47]
CD309 (Flk-1; VEGFR-2)	Bone marrow	[78]
CD349 (frizzled-9)	Placenta	[18]
ALDH	Bone marrow	[36]
GD2 (neural Ganglioside)	Bone marrow	[79]
	Umbilical chord	[21]

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Table 2 (continued)

Markers used	Tissue	References
HSP90beta	Bone marrow	[80]
Integrin alphaV/beta5	Bone marrow	[57]
SSEA-4	Bone marrow	[81]
TNAP	Bone marrow	[7, 28–30]
(b) *Unknown (antibody-defined) antigens for positive selection*		
3G5	Adipose tissue	[17]
D7-FIB	Bone marrow	[76, 82]
STRO-1	Bone marrow	[30, 51, 54, 69, 70, 83–89]
	Adipose tissue	[17]
W5C5	Bone marrow	[38]
(c) *Known antigens for negative selection*		
CD3 (T cell surface glycoprotein)	Peripheral blood	[20]
CD14 (LPS receptor)	Peripheral blood	[20]
CD31 (PECAM-1)	Bone marrow	[78]
	Adipose tissue	[23–25]
CD34 (hematopoietic progenitor cell antigen)	Bone marrow	[30, 78, 89–91]
	Peripheral blood	[20]
CD45 (leukocyte common antigen)	Bone marrow	[51, 56, 64, 76, 83, 89, 90, 92]
	Lung	[19]
	Adipose tissue	[24]
CD105 (endoglin)	Adipose tissue	[24, 25]
CD144 (cadherin-5)	Adipose tissue	[23]
CD146 (MCAM)	Adipose tissue	[24, 25]
CD235a (glycophorin A)	Bone marrow	[51, 83, 85, 89, 92]
Lin⁻ (various antigens)	Bone marrow	[34, 65, 92]

3.3 Prospective Isolation of MSC Subsets

Several groups have reported that MSCs are heterogeneous with respect to their growth and differentiation potential [35, 39, 40]. However, little information exists about markers that discriminate between developmentally, functionally, and morphologically distinct MSC subsets. Recently, we introduced a monoclonal antibody against CD56 that recognizes a distinct MSC subset with high selectivity [7]. This antibody (termed 39D5) detects a CD56 epitope, which is not expressed on NK cells (Fig. 3a) but is highly expressed on about 0.5–15 % of CD271^{bright} cells (Fig. 3b). Giemsa staining revealed that CD56⁻ cells contain a large bright cytoplasm with vacuoles, whereas cells of the CD56⁺ subset contain a smaller cytoplasm with basophilic granules (Fig. 3d). Interestingly, cells of the CD56⁺ population were about two times more clonogenic than cells of the CD56⁻ subset (Fig. 3c). Further analysis has shown that this increased frequency of clonogenic cells correlates with an increased proliferation rate [7]. Surface marker expression analysis of sorted CD56⁺ and CD56⁻ cells revealed that only CD56⁺ cells but not
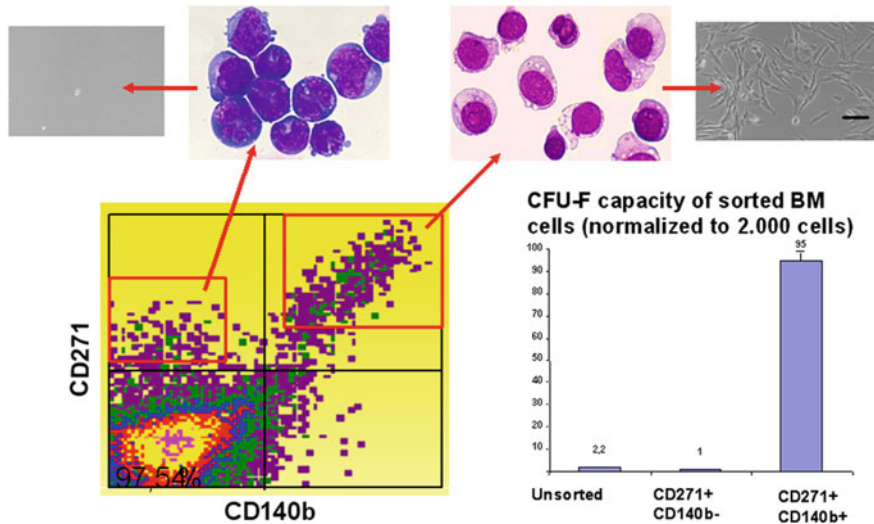
Fig. 2 Morphological features and clonogenic capacity of sorted CD271^{bright}CD140b^{+} and CD271^{dim}CD140b^{−} bone marrow cells. Cells were stained with anti-CD271 and anti-CD140b, gated on the indicated populations, and sorted by flow cytometry. Fourteen days after culture in serum-free, b-FGF containing medium, the resulting colonies were enumerated and CFU-F numbers normalized to 5.000 plated cells. Note that CFU-F were exclusively found in the CD140b^{+} subset and only cells of this subset gave rise to fibroblast-like cells. The morphology of CD271^{dim} and CD271^{bright} cells was evaluated by staining of sorted cells with Giemsa

CD56^{−} cells coexpress CD166, and only a subset of CD56^{−} cells but not CD56^{+} cells express CD349 (frizzled-9) [7]. When cultured cells of both populations were induced to differentiate into defined cell lineages, only MSCs derived from the CD56^{−} population were able to differentiate into adipocytes [7]. In contrast, only MSCs from the CD56^{+} subset effectively gave rise to chondrocytes, suggesting that this subpopulation is the preferred source for therapeutic approaches in the field of cartilage tissue repair [7].

We have previously shown that CD56^{+} MSCs express low levels of TNAP, a molecule that is upregulated during osteogenic differentiation [7]. In a model proposed by Gronthos et al., cell surface expression of TNAP is absent on early STRO-1^{+} stem cells but upregulated during osteogenic differentiation [41]. This STRO-1^{+}TNAP^{−} population may correspond to the recently described CD56^{+}TNAP[−/dim] subset, which was identified by our group. In agreement with this hypothesis, cells of the CD56^{+} subset mature at a later time point into osteoblasts compared to CD56^{−} cells. We therefore propose an extended model, in which STRO-1^{+}CD56^{+}TNAP[−/dim] MSCs represent an immature precursor with multi-lineage differentiation capacity. Cells committed to the chondrocyte lineage diverge at very early (CD56^{+}) stages of MSC differentiation. This chondrogenic potential, which is rapidly lost upon differentiation into TNAP^{+}CD56^{−} cells, is accompanied by the induction of the adipogenic differentiation potential.
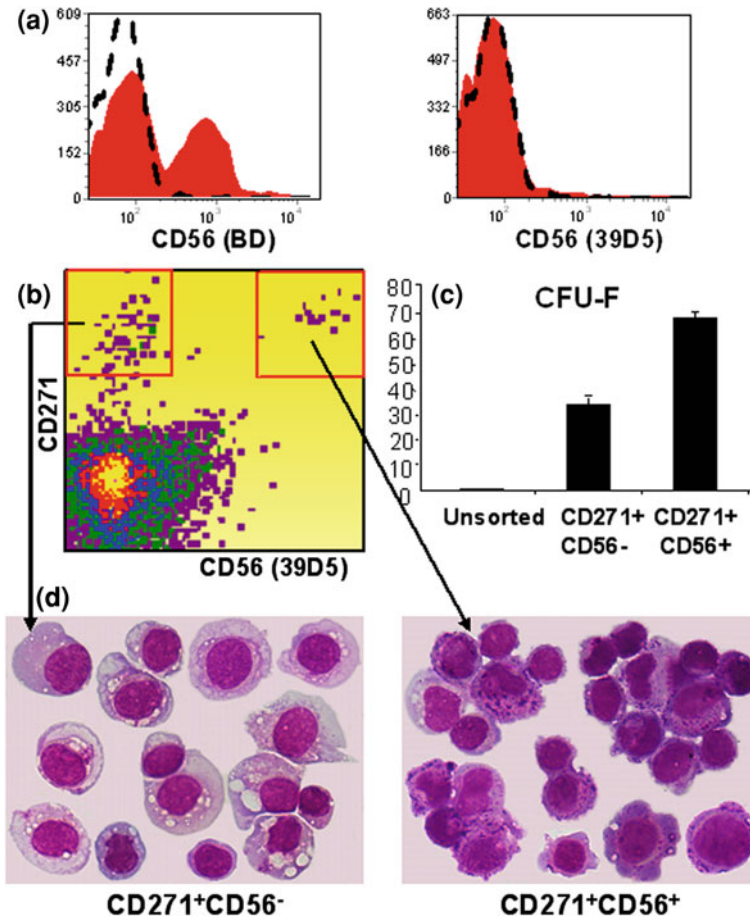
Fig. 3 CD56 defines a subset of MSC. **a** CD56 epitope NCAM16.2 but not 39D5 is expressed on natural killer cells from peripheral blood. **b** CD56 is expressed on cells of a CD271^{bright} MSC subset. **c** CD271^{bright}CD56$^+$ and CD271^{bright}CD56$^-$ BM cells are clonogenic. CFU-F derived from 500 FACS-sorted cells was stained and scored as described. Data represent the mean CFU-F numbers from three different experiments (*$p < 0.01$). **d** Morphology of CD271^{bright}CD56$^-$ and CD271^{bright}CD56$^+$ cells. Subsets were sorted, cytocentrifuged, stained with Giemsa solution, and scored on a Zeiss Axiovert 200 microscope. Note the presence of basophilic-like granules in cells of the CD271^{bright}CD56$^+$ population

Several reports underline the important role of CD56 expression on fibroblasts to support the growth of hematopoietic stem cells [42–44]. The contribution of CD56 was initially described in mouse and monkey models [42, 43], but a more recent report showed that CD56 expressed on a mouse stromal line plays a crucial role to support human hematopoiesis in vitro and in vivo [44]. The authors showed that co-culture of CD34$^+$CD38$^-$ cord blood cells with a CD56$^+$ stromal cell line resulted in a significantly greater expansion rate of CD34$^+$ hematopoietic cells
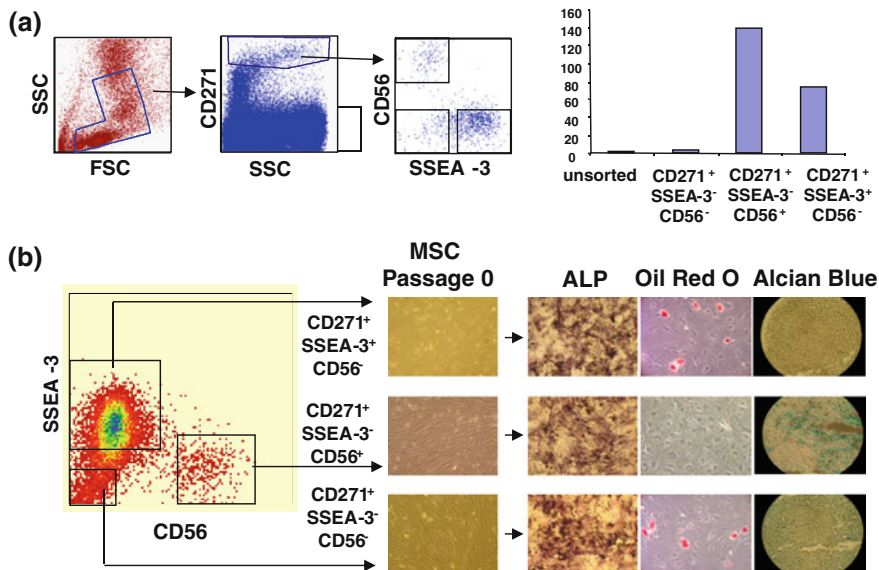
Fig. 4 a CD56 and SSEA-3 define two distinct MSC subsets. **a** CD56 and SSEA-3 define two distinct MSC subsets. MSCs express either CD56 or SSEA-3. The frequency of CFU-F in the CD56$^+$ population is about two times higher compared to the SSEA-3$^+$ population. **b** Only SSEA-3$^+$ but not CD56$^+$ MSCs are able to differentiate into Oil Red O-positive adipocytes. In contrast, CD56$^+$ but not SSEA-3$^+$ MSCs give rise to Alcian Blue-positive chondrocytes

compared to stromal cells, which did not express CD56. This enhancing effect could be blocked by the addition of an inhibitory anti-CD56 antibody, suggesting that direct interactions between CD56 molecules from different cells are essential. It remains unclear whether CD56 on human MSCs plays a similar hematopoiesis-supporting activity. Because human hematopoietic stem cells do not express CD56, it is unlikely that a potential supporting effect is caused by homotypic interactions between CD56 molecules on stromal and hematopoietic cells. Rather, CD56$^+$ stromal cells may interact with extracellular matrix components, such as heparin sulphate or chondroitin sulphate proteoglycans.

We have recently shown that SSEA-3 (but not SSEA-4, TRA-1-60, or TRA-1-81) is a candidate marker for MSCs from primary femur-derived bone marrow [28]. To verify this assumption, bone marrow cells were stained with CD271, SSEA-3, and CD56 and gated on CD271^{bright}SSEA-3$^-$CD56$^-$, CD271^{bright}SSEA-3$^+$CD56$^-$, and CD271^{bright}SSEA-3$^-$CD56$^+$ cells (Fig. 4a). The clonogenic potential and the differentiation capacity of the sorted populations were determined by CFU-F assays and appropriate differentiation protocols. As shown in Fig. 4a, clonogenic cells were about 44-fold enriched for CFU-F in the CD271^{bright}SSEA-3$^+$CD56$^-$ population, 83-fold in the CD271^{bright}SSEA-3$^-$CD56$^+$ population, but only about 2-fold in the

CD271^{bright}SSEA-3$^-$CD56$^-$ fraction. Not surprisingly, CD271^{bright} SSEA-3$^+$CD56$^-$ MSCs gave rise to osteoblasts and adipocytes, but not to chondrocytes. In contrast, CD271^{bright}SSEA-3$^-$CD56$^+$ cells were able to differentiate into chondrocytes but not into adipocytes (Fig. 4b). Collectively, SSEA-3 is a suitable and selective marker for the isolation of adipocyte precursors, whereas CD56 is a more appropriate target for the isolation of chondrocyte precursors.

Many of the tested markers in both subsets are either upregulated or downregulated during culture (Table 1). Although CD271 is expressed at high levels in all primary MSC subsets, and CD56 and SSEA-3 are expressed in the respective subsets, these antigens are rapidly downregulated during culture. CD166 is expressed at low levels on primary CD56$^+$ MSCs but upregulated to high levels on all cultured MSCs. Finally, CD109 and CD318 are negative on primary MSCs and expressed at high levels on cultured MSCs. These data suggest that the conventional definition of MSC-reactive surface markers, which is based on cultured cells, may be revised and specified.

Tormin et al. described the localization of two MSC subsets in distinct areas of the bone marrow: dominating perivascular MSCs that coexpress CD271 and CD146, as well as bone-lining MSCs that express only CD271 but not CD146 [45]. This prompted us to analyze whether the bone-lining MSCs correspond to the CD56$^+$ MSC subset and whether the perivascular CD146$^+$ MSCs lack CD56 expression. We not only confirmed that MSCs in perivascular regions coexpress CD271 and CD146, but also showed that CD271$^+$ bone-lining MSCs are negative for CD146 and positive for CD56 (manuscript submitted). This suggests that, apart from the distinct surface antigen expression profile, the distinct morphology, and differentiation potential, bone-lining MSCs may have also distinct functional properties not yet identified. As the niches of CD34$^+$ hematopoietic stem cells are supposed to be localized near the bone, it is intriguing to speculate that CD56$^+$ but not CD56$^-$ MSCs contribute to the stromal niche of CD34$^+$ hematopoietic stem cells.

3.4 Isolation of a Single MSC

We have recently described the prospective isolation of MSC subsets from primary tissue using antibodies against molecules, which are selectively expressed on the surface of these subsets [7, 29]. Although phenotypically distinct MSC subsets exhibit properties that are unique with regard to their proliferation and differentiation capacity, there is still a broad heterogeneity at the clonal level [7]. Heterogeneity of individual MSC clones has been reported by several groups, who demonstrated that the developmental and proliferative potential was highest in cells giving rise to large colonies, whereas small-sized colonies were derived from cells with limited differentiation and proliferation capacity [7]. Sorting of single cells into culture plates does not only provide information about the growth characteristics of individual MSC clones but also about the frequency of MSC clones with defined differentiation potential. Pittenger et al. described that almost

100 % of colonies derived from single bone marrow cells underwent osteogenic differentiation; about 80 % of the colonies revealed adipogenic differentiation potential, but only 30 % of the colonies showed chondrogenic differentiation potential [5]. Our group successfully isolated clones with the capacity for osteoblasts but not adipocyte differentiation, as well as for adipocytes but not osteoblast differentiation [7]. Further experiments are required to determine the frequency of MSCs with multipotent differentiation capacity as well as MSCs with restricted differentiation potential. These analyses may contribute to customized complex models of MSC maturation and differentiation, similar to those proposed for cells of the hematopoietic system.

4 Concluding Remarks

Conventionally, MSCs are functionally isolated by their capacity to adhere to the surface of culture plates. The resulting cells are poorly defined and give rise to a heterogeneous mixture of cells including MSCs, reticular cells, macrophages, and endothelial cells. To gain information about the starting population, several markers have been introduced to prospectively isolate and characterize MSCs and their subsets. A similar degree of hierarchy and progenitor cell heterogeneity may exist among MSCs as described for the hematopoietic system. The identification of MSC subsets and clonal analysis of individual MSCs may provide more insight on the heterogeneity of MSCs from different tissues.

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Urine as a Source of Stem Cells

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Abstract Traditionally, clinicians and researchers have relied on a skin biopsy or blood extraction as relatively accessible supplies for in vitro cell expansion and biological studies. Perhaps surprisingly, limited attention has been given to a totally noninvasive source, urine, which eliminates the discomfort associated with other procedures. This may arise from the perception that urine is merely a body waste. Yet, the analysis of urine is a longstanding fundamental test for diagnostic purposes and nowadays there is growing interest in using urine for detecting biomarkers. In addition, recent work including ours reinforces the idea that urine contains a variety of viable cell types with relevant applications. In this review, we describe those cell types and their potential uses.

Keywords Adult stem cells · Induced pluripotent stem cells · Reprogramming · Tissue engineering · Urinary cells · Urine

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Abbreviations

EGF   Epidermal growth factor
ESCs   Embryonic stem cells
iPSCs   Induced pluripotent stem cells
USCs   Urine-derived stem cells

Contents

1 Urine as a Cell Source

The human urinary tract comprises a highly sophisticated tubular network whose total surface is bigger than the skin [1]. It is composed of two kidneys containing myriad renal tubules, two ureters, the urinary bladder, and the urethra (Fig. 1). It converts the continuous involuntary production of filtrate by the kidneys into the intermittent and consciously controlled voiding of urine by the urethra. This ensures that harmful products in the blood are regularly removed and excreted, and also helps maintain adequate blood pressure and the acid–base equilibrium [2]. The basic structural and functional unit of the kidney is the nephron, which consists of the renal corpuscle (responsible for filtering blood) and the renal tubules (Fig. 1). Together, the two kidneys produce ∼180 l of primary filtrate every day, of which only 1–2 l are finally excreted as urine [3]. Not unexpectedly, given the large dimensions of the tubular network and the shear stress produced by the glomerular filtrate, many viable and nonviable cells (up to 7,000 daily) detach from all surfaces along the urinary tract and can be collected in urine [4]. Because these luminal surface linings are all epithelial, the human urine sediment is a major source for epithelial cells [4, 5]. These include renal tubular cells (from the proximal, distal, and convoluted tubules, and the collecting duct), transitional epithelial (also termed urothelial) cells from different downstream locations in the urinary tract (renal pelvis, ureters, bladder, glandular ducts of the prostate and the proximal urethra), and squamous cells from the distal urethra (Fig. 1). In contrast to the renal epithelium, the urothelium is stratified and contains three different cell types: basal cells (a single layer), intermediate cells (a multilayer), and umbrella
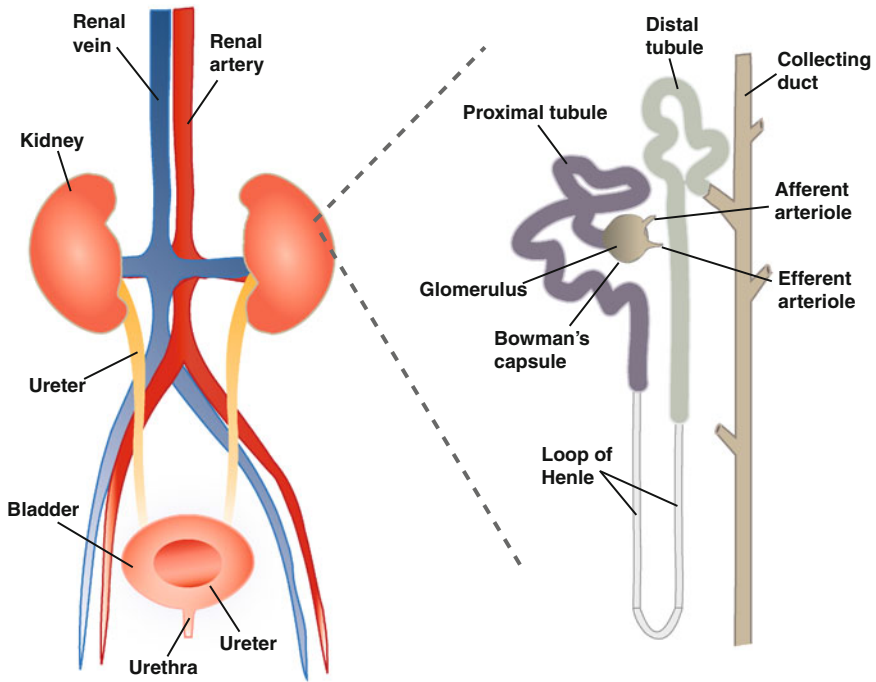
Fig. 1 Structure of the human urinary tract and the nephron. A simplified view of the urinary tract apparatus is shown on the *left*, which consists of two kidneys, two ureters, the bladder, and the urethra. The structure of a nephron is shown magnified on the *right*. Each nephron consists of the renal corpuscle (glomerulus and Bowman's capsule) and the renal tubules (proximal convoluted tubule, loop of Henle, and distal convoluted tubule)

cells (a single layer). Voided urine may also contain squamous cells derived from the similarly stratified epithelia of the vulva, vagina, uterine, and cervix. Accordingly, squamous cells are more abundant in the urine sediment of females and their amount varies depending on the hormonal status of the woman [6, 7]. Cells derived from blood such as erythrocytes, leukocytes, and macrophages can also be observed in urine, and their presence increases in aged individuals or due to disease.

Sutherland and Bain first reported the successful isolation of viable cells from urine using samples of newborn infants [8]. In the following years, the procedure was reproduced by multiple groups [9, 10], and cells were also collected from patients with various diseases, including diabetes mellitus, nephropatic cystinosis, and acute tubular necrosis [11–13]. These urinary cells displayed different morphologies (either polygonal or more elongated) but their origin was shown to be mostly epithelial (from the kidney tubules and urothelial) based on the expression of marker genes [4]. Nonetheless, fibroblast-like populations have also been reported [4, 14]. Primary (urinary) epithelial cells from healthy individuals are of great interest for toxicological research [15]. In addition, urinary cells from patients with genetic renal
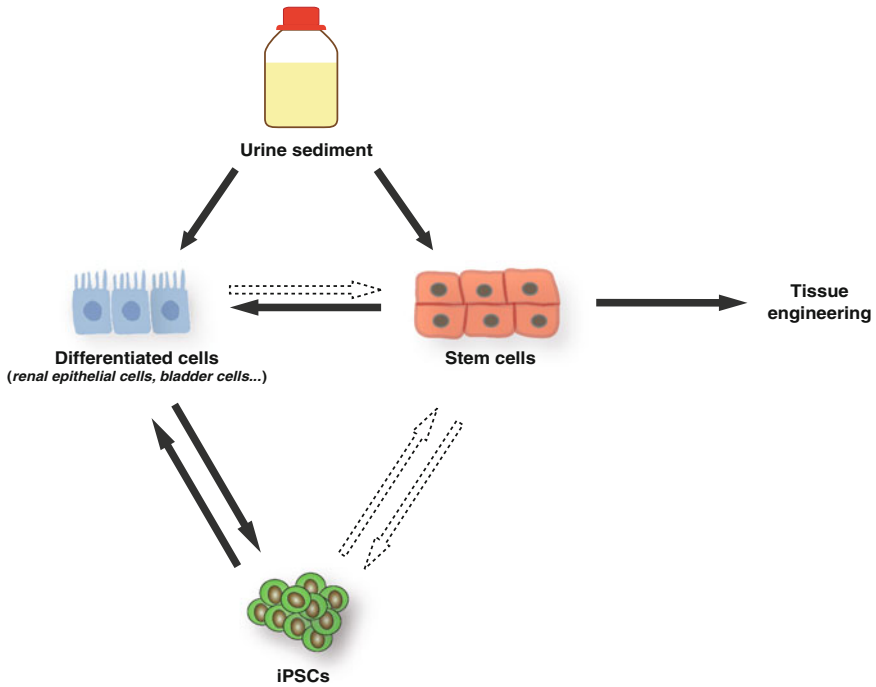
Fig. 2 Urine as a source of cells. The human urine sediment consists of differentiated cells, such as renal epithelial cells, urothelial cells, and stem cells (USCs). USCs can be differentiated into mature cells of different lineages. Thus, USCs are of great interest for cell-based tissue-engineering approaches. Differentiated cells, and likely USCs as well, can be used for reprogramming to iPSCs, and these cells have the potential to produce all cell types of the mammalian body including kidney cells and potentially also USCs. Direct reprogramming of differentiated cells to USCs may be feasible by direct dedifferentiation using lineage instructive transcription factors

conditions are potentially helpful to understand the disease mechanisms, as they may include populations that display loss of heterozygosity or have acquired additional mutations [16]. Hence, these early reports offered an attractive cell source whose utility warranted further investigation (Fig. 2).

2 Urine as a Source of Stem Cells

2.1 Urine as a Source of Adult Stem Cells

Adult (also termed somatic) stem cells can be found in various tissues throughout the human body [17]. Upon local injury or due to physiological turnover, they have the unique ability of transforming into some or all the cell types that compose a

particular organ. This property to generate diverse, but restricted, specific cell types is called multipotency, and contrasts with pluripotency, which is normally applied to reflect the ability of embryonic stem cells (ESCs) to produce all cells that compose the mammalian body [18]. Adult stem cells are of significant interest for cell therapy and tissue-engineering applications because of their potential for self-regeneration and autologous transplantation. Moreover, in some cases, they have immunomodulatory characteristics that facilitate the engraftment of heterologous tissues [19].

The first adult stem cells were isolated from the bone marrow, but in recent years similar populations have been found in almost any organ [20]. A general molecular signature for adult stem cells of different tissues has not yet been identified and each organ seems to use independent sets of transcription factors [21, 22]. Nonetheless, adult stem cells share in common a close relationship with their surrounding environment (usually referred to as the *niche*), which is responsible for sustaining or altering the balance between *self-renewal* (the ability to divide and remain undifferentiated) and differentiation [23]. The latter possibly determines that most adult stem cells have been challenging to expand or maintain in culture [17]. This, together with the reduced endogenous availability and the requirement of an invasive procedure for extraction, represents a major caveat.

Recently, Zhang and colleagues [14] have demonstrated that human urine also contains a stem cell population (termed urine-derived stem cells or USCs), which can be expanded in vitro up to ten passages (Fig. 2). Few USCs ($\sim$7) are normally contained in 100 ml urine, but they can reach 4 million at passage 4 using media with a high concentration of epidermal growth factor (EGF) [14]. USCs express CD44, cytokeratin 13, and uroplakin, all of which are also present in basal urothelial cells [14]. Accordingly, they are thought to be derived from the latter cell type. Inside the body, basal cells progressively give rise to intermediate and umbrella cells, but the turnover is slow ($\sim$3–6 months) compared with other epithelia (e.g., the gut). Yet, basal cells show an enormous regenerative capability upon damage [24, 25], perhaps explaining why it is relatively easy to collect and expand them using urine. USCs also display mesenchymal stem cell and pericyte markers such as CD73, CD105, and CD146, but are negative for hematopoietic or endothelial markers including CD45, CD34, and CD31 [14]. Moreover, they can differentiate into multiple bladder lineages (e.g., urothelial, smooth muscle, endothelial) and other mesodermal cell lineages, such as chondrocytes, adipocytes, and osteocytes [26]. Zhang and colleagues made the first attempts to use USCs for tissue engineering [26–28]. Among these stands the generation of a tissue-engineered urethra by seeding USCs on a three-dimensional porous small intestinal submucosa scaffold [26]. The authors also isolated USCs from the upper urinary tract through a biopsy [29], which it is rather expensive but relevant because in some circumstances (e.g., urinary tumors) cancer cells may contaminate urine samples. Therefore, urine is an unexpected source of stem cell-like cells that can be easily expanded in vitro.

2.2 Urinary Cells as Donor Cells for Producing iPSCs

During development, the morula progressively forms all the specialized cell types of a mammalian body in a one-way process of epigenetic commitment that was thought to be irreversible [30]. In this context, for example, a heart cell does not naturally turn into a skin cell, nor does a brain cell give rise to a liver cell. However, the maintenance of tissue-specific identity is steadily challenged by both endogenous and exogenous (environmental) factors. This causes, among other things, multicellular organisms to have a high risk of suffering cancer during a lifetime, as this is in fact a process of cellular dedifferentiation that shares a striking similitude with embryogenesis [31].

The first direct demonstration of the plasticity of cell fate came from early studies in frogs by Briggs and King [32], who transferred the nucleus of a somatic cell into an enucleated oocyte to produce normal swimming tadpoles of *Rana pipiens*. This procedure was named somatic cell nuclear transfer (or nuclear transfer) and culminated almost half a century later with the cloning of Dolly the sheep [33]. These discoveries paved the way for a field of study termed nuclear reprogramming or more simply reprogramming, which moved in parallel with the isolation and study of first mouse [34, 35] and then human ESCs [36]. Reprogramming to an ESC-like stage was likewise achieved by fusing somatic cells with ESCs [37, 38], altogether reinforcing the idea that the nuclei of both the egg and ESCs contain factors responsible for changing cell fate [30, 39]. Based on these principles, Takahashi and Yamanaka reported in 2006 the transformation of mouse fibroblasts into ESC-like cells by overexpression of four transcription factors (Sox2, Klf4, Oct4, and c-Myc) highly enriched in the latter cell type [40]. These cells were termed induced pluripotent stem cells or iPSCs. The procedure was subsequently reproduced by several groups using human cells from normal individuals and also a multitude of patients with genetic diseases [41–46]. Remarkably, iPSCs are highly similar to ESCs morphologically, functionally, transcriptionally, and at the level of genomewide distribution of chromatin modifications [41, 47–50]. Given that they share the ability to differentiate into all tissues that compose the mammalian body [51], iPSCs hold great hope for regenerative medicine, toxicity screening, and disease modeling [52, 53].

Human iPSCs have thus far been generated using a large variety of donor cells from different tissues. These include skin (fibroblasts, keratinocytes, and melanocytes), blood (peripheral and from the umbilical cord), adipose tissue, periosteum and periodontal ligament, neural cells, and glia, hepatocytes, amniocytes, and cells from extraembryonic tissues (umbilical cord matrix and the placenta) [41, 42, 54–70]. However, producing iPSCs from one donor cell type or another is not irrelevant due to multiple considerations. First, cells of different origins may require different reprogramming methodologies (some of them not always trivial) and produce iPSCs with different kinetics and efficiency [71]. In this regard, when choosing one procedure and cell source or another, simplicity, speed, and affordability should prevail if there is no detriment for the quality and reproducibility. Second, there is a growing body of evidence that reprogramming leaves a
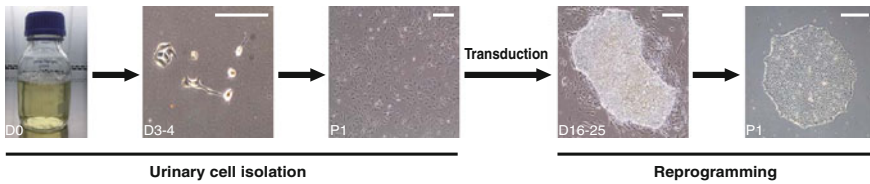
Fig. 3 Urinary iPSC generation. Schematic representation of the kinetics of urinary cell isolation and reprogramming. Phase contrast photographs for representative stages are shown. Urinary cell colonies routinely appear within the first 4 days of culture and can be readily expanded for subsequent reprogramming. iPSC colonies usually appear between 16 and 25 days after transduction with the exogenous factors. *D* day; *P* passage. Scale bars = 200 μm

series of scars in the genome (somatic mutations and copy number variations) and epigenome (epigenetic aberrations and memory of the donor tissue) [72–76]. These alterations may pose a risk for cell-based therapies and faithful in vitro disease modeling [43, 52]. Therefore, although at the beginning there was a general obsession for increasing reprogramming efficiency, it is now clear that producing clones with fewer abnormalities is more important [77]. Third, undergoing an invasive procedure for donating samples is frequently rejected, and noninvasive materials such as extraembryonic tissues are only available after birth unless properly stored.

Based on these considerations, a promising universal cell source for iPSC generation is peripheral blood [78], as it can be easily obtained with minimal invasion and stored frozen for a long time before reprogramming. Yet, in rare cases such as religious beliefs, severe immunodepression, or infection, blood may not be a feasible option. Extraction of hair follicles is also not strictly noninvasive and even though the procedure seems simple it may require multiple trials [79]. In addition, there are reports describing epigenetic memory of the donor tissue for iPSCs of these two origins [72, 80], although this may actually be a problem for any donor cell type.

Recently, we demonstrated that urine samples can reproducibly be used as an efficient cell source for producing iPSCs [81]. We have thus far generated urinary iPSCs from over 27 individuals (healthy and diseased) using an integrating method (retroviral delivery). Compared with other procedures, urinary iPSC generation is affordable (as the only cost is the culture medium) and highly reproducible. A single sample collection of 30–50 ml normally yields sufficient urinary cells for iPSC generation after only 2 weeks of culture (Fig. 3), which is not substantially longer compared to a skin biopsy or hair follicle extraction. Urinary iPSCs normally appear as early as 2–3 weeks post transduction with the four Yamanaka factors [40] (Fig. 3), and they routinely show excellent differentiation potential after colony picking and expansion [81]. Efficiencies of colony formation range from 0.01 % for cells from a 65-year-old up to 4 % from a younger individual, which is at the very least comparable to optimal reprogramming efficiencies achieved by others using fibroblasts or blood. Moreover, frozen urinary cell samples can also be reprogrammed but there is a drop in efficiency.

In our experience, the starting urinary cell population seems to have a predominant renal tubule origin but other cell types are likely included [81]. Therefore, we cannot exclude that urinary iPSCs also arise from other cell types including the USCs described by Zhang and colleagues [14, 29]. The latter should not represent a problem for disease modeling, as we have not observed any specific bias for tissue-specific differentiation among iPSCs from multiple donors [81]. However, for studies on genomic alterations or epigenetic memory it may be convenient to sort out specific cell populations before performing the reprogramming [82, 83]. In this regard, it is interesting to speculate that iPSCs produced from urine may be more easily differentiated into kidney or kidney progenitor cells than iPSCs of other origins [72] (Fig. 2). Related to this, two additional groups have reported the generation of iPSCs from kidney samples obtained through renal biopsy: mesangial cells and renal proximal tubular epithelial cells [84, 85]. It will be relevant to study whether kidney iPSCs from these three independent sources have similar characteristics and whether the putative existence of epigenetic memory can be used to improve existing protocols of kidney differentiation [86]. In summary, urine represents a novel source for producing human iPSCs that has a number of advantages compared to others.

3 Conclusions

There is a growing view that the utility of urine for biomedical purposes may have been underestimated. It is, for example, an increasingly relevant source of biomarkers for a wide range of diseases, and not only renal diseases [11–13]. Work by Zhang and colleagues has also demonstrated that urine is a novel source of stem cells [26–28] (Fig. 2). The full potential of these USCs for tissue engineering remains yet to be explored but the preliminary results are encouraging. Recently, we showed as well that urinary cells are an optimal supply for producing iPSCs [81] (Fig. 2). The reprogramming of urinary cells to iPSCs is simple, affordable, and completely noninvasive. Moreover, it is universal (it can be applied to any gender, race, or age) except for those rare cases with renal insufficiency and dialysis, or oncological patients with cystectomy. In renal patients undergoing dialysis, the peritoneal fluid may still be collected and used for reprogramming purposes, as it is known to contain viable cells that grow readily in vitro [87]. Additional studies should be performed to discern whether the genome and epigenome of urinary iPSCs is less corrupted than iPSCs from other donor cell types. One argument supporting such an idea is that urinary cells are naturally less exposed to solar radiation than the skin, but it is yet unclear which cell type displays higher genomic stability in vitro. Of relevance, the comparisons should be done with samples obtained from the same individual that have been grown for the same number of passages before reprogramming. Modifications of the tissue culture conditions before and during the reprogramming, for example, use of hypoxia or antioxidants, may also be determinant [61, 88]. Likewise, for clinical

purposes the exogenous factors should be delivered using nonintegrating vectors such as episomes or modified RNAs [89, 90]. From a different perspective, it will be interesting to study whether urinary cells are also susceptible to other forms of nuclear reprogramming, for example, transdifferentiation into different epithelia (e.g., liver cells) [91, 92].

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Expansion of Mesenchymal Stem/Stromal Cells under Xenogenic-Free Culture Conditions

Sven Kinzebach and Karen Bieback

Abstract Mesenchymal Stem/Stromal cells (MSCs) are increasingly applied in cell-based regenerative medicine. To yield clinically relevant cell doses, ex vivo expansion of MSCs is required to be compliant with good manufacturing practice (GMP) guidelines. A lack of standardization and harmonization seems to hamper rapid progress in the translational phase. Most protocols still use fetal bovine serum (FBS) to expand MSCs. However, the high lot-to-lot variability, risk of contamination and immunization call for xenogenic-free culture conditions. Chemically defined media are the ultimate achievement in terms of standardization. These media, however, need to maintain all key cellular and therapy-relevant features of MSCs. Because of the numerous constituents of FBS, the development of such chemically defined media with an optimal composition of the few essential factors is only beginning. Meanwhile, various human blood-derived components are under investigation, including human plasma, human serum, human umbilical cord blood serum and human platelet derivatives such as platelet lysate.

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Contents

1 Mesenchymal Stem/Stromal Cells

Regenerative medicine is characterized by a fast-growing interest in biomedical research. The aim is to repair, regenerate or replace cells, tissues or organs via cell-based therapies. These can be based either on the stimulation of endogenous regeneration and repair processes or on the application of ex vivo cultured cells, including mature, progenitor or stem cells. In the 1980s the focus was on ethically debated embryonic stem cells, so the discovery rapidly changed interests towards this cell type of adult human stem cells [100]. Mesenchymal stem/stromal cells (MSCs) were the first nonhematopoietic progenitors isolated from the bone marrow (BM) in the 1970s by Friedenstein et al. [35, 36]. The authors previously described the multilineage differentiation potential for a variety of mesodermal lineages, such as bone, cartilage, fat, marrow stroma, tendon, muscle, dermis, and connective tissues. However, the field seemed to rest until work in the 1990s turned researchers' attention again to MSCs [19, 76]. Analogous to the hematopoietic stem cell (HSC), the term "mesenchymal stem cell" was introduced [18]. However, because later studies failed to fulfill the self-renewal criterion of stem cells (i.e., self-renewing, unspecialized and having differentiation capacity to specialized cell types), the term "mesenchymal stromal cell" was suggested instead [43]. Of note, MSCs are characterized as culture-adapted, ex vivo expanded cells. This population is heterogeneous, containing progenitor cells at different maturation stages and also mature stromal cells [73]. The heterogeneity of cell preparations, the use of different tissues as starting materials and differing isolation and cultivation protocols make comparability complicated. In an effort to standardize terms, the International Society for Cell Therapy (ISCT) defined minimal criteria to be fulfilled by MSC [27]:

- Adherence to cell culture plastic surfaces accompanied by a fibroblastoid phenotype
- Expression of typical markers (CD105, CD73, and CD90) and lack of expression of CD45, CD34, CD14 (or CD11b), CD79α (or CD19) and HLA-DR surface molecules, and
- Differentiation towards at least the three mesodermal chondrocyte, adipocyte, and osteocyte lineages.

2 Clinical Application: Balancing Success and Risks

2.1 Success

Broad mesodermal differentiation potential led to early trials in diverse clinical fields, including bone and cartilage repair as well as cardiovascular and neurological diseases [2, 8, 44]. Other studies focused on using stromal support capacity to facilitate HSC engraftment [53, 60]. The discovery of long-lasting therapeutic efficacy (despite an **unexpectedly** low level of engraftment) was **unexpected** MSC researchers then went "back to the bench" to answer the question of how MSCs achieve this therapeutic benefit without actually being present [77]. Seminal studies finally revealed that MSCs inherit strong immunomodulatory properties [61, 59]. In combination with their low immunogenicity [94], this makers MSCs well-suited for both autologous and allogeneic transplantation settings. The observed beneficial therapeutic effects were subsequently further attributed to the capacity of MSCs to target sites of inflammation and injury. Within such an environment, MSCs release a variety of pro-regenerative, anti-apoptotic, and anti-fibrotic factors that enable endogenous repair processes [20].

In summary, the combination of all these activities—differentiation capacity, hematopoietic support, immunomodulatory, and pro-regenerative features—account for the promising therapeutic potential of MSCs.

Accordingly, MSCs are increasingly applied in cell-based therapy: currently, 198 clinical trials are found by searching http://clinicaltrials.gov (for "mesenchymal stem cells" and 20 trials for "mesenchymal stromal cells" accessed 02/2012). There are also numerous reports on preclinical data. A variety of disease entities are characterized by inflammatory reactions and tissue degeneration. As indicated previously, in most settings the combined action of MSC characteristics results in successful therapy. The majority of preclinical and clinical data report no adverse events after MSC application, indicating that MSCs can be applied safely. However, the heterogeneity of the tissue source used as starting material and the diverse protocols for isolation and cultivation have been hampering further progress in their clinical applications. As a precaution, universal standards and harmonized protocols need to be implemented to ensure safety and efficacy.

2.2 Risks

Although current data generally indicate that MSCs can be used safely, a few adverse events have been reported. The transplantation of MSCs into infarcted hearts has caused calcifications, possibly due to osteogenic differentiation events [14]. MSC infusion has been demonstrated to facilitate tumor growth [51, 79]. Importantly, latent tumors (such as gliomas, sarcomas and melanomas) and also metastases manifested after MSC infusion, indicating that tumor surveillance may be impaired by the immunosuppressive activities of MSCs [57]. It is also possible that MSCs directly favor tumor growth by integrating into the tumor stroma and secreting angiogenic factors [51].

It has been intensely debated whether the ex vivo expansion of MSCs, which in most cases is needed to achieve clinically relevant cell numbers, induces spontaneous transformations [62, 78]. Human MSCs generally undergo replicative senescence. Cells depict typical cellular changes, shortening of telomeres, an irreversible cell cycle arrest, loss of differentiation potential and resistance against apoptosis [15, 23, 55]. Imbalances in the tightly regulated system of proliferation and cellular aging/senescence can be momentous, and accumulated genetic and/or epigenetic changes can cause transformation [89]. Immortalized MSCs that are either spontaneously transformed or induced to express telomerase reverse transcriptase (hTERT) have numerous genetic and epigenetic alterations. Karyotype aberrations are not necessarily observed [17]. Aneuploidy, although observed in few clinical-scale MSC preparations, failed in all cases to cause transformation. All samples underwent progressive growth arrest and senescence [95].

To ensure safety and efficacy, all steps within the MSC manufacturing process need to be standardized. Cellular quality and potency have to be reproducible. Thus, it seems necessary to define common standards and to harmonize protocols.

2.3 Regulatory Frameworks

Translating MSCs into cell therapy settings requires formal approval by a regulatory authority. The manufacturing process and manufacturing authorisation have to comply with the current national regulatory framework. Including current good tissue practice (GTP), good laboratory practice (GLP), good manufacturing practice (GMP) and good clinical practice (GCP) guidelines. These guidelines are set to control the safety, purity and potency of therapeutics and are related to the complete manufacturing process: facilities, personnel, equipment, reagents, supplies, and procedures (tissue procurement, cell isolation, selection, expansion, quality control, release, transport, clinical application, etc.). Procedures need to be validated according to the criteria defined by the manufacturer. When MSCs are to be used in a medicinal product, the donation, procurement and testing of the cells are covered in Europe by the Tissues and Cells Directive (2004/23/EC). To make

innovative treatments available to patients and also to ensure that these novel treatments are safe, the EU approved the "Regulation on Advanced Therapies" (EC1394/2007). This regulation defines products as "Advanced Therapy Medicinal Products (ATMP)" if they are:

- "A gene therapy medicinal product" (Part IV of Annex I to Directive 2001/83/EC)
- "A somatic cell therapy medicinal product" (Part IV of Annex I to Directive 2001/83/EC) and
- "A tissue engineered product".

Cells or tissues fulfilling at least one of the following conditions are considered to be "engineered":

- "The cells or tissues have been subject to substantial manipulation, in order to unfold their biological characteristics, physiological functions or structural properties" or
- "The cells or tissues are not intended to be used for the same essential function or functions in the recipient as in the donor" (Official Journal of the European Union 10.12.2007).

The quality system for U.S. Food and Drug Administration (FDA)-regulated products is known as current good manufacturing practices (cGMP). Globally operating pharmaceutical facilities have to fulfil the requirements of both the FDA and EU. The Code of Federal Regulation (CFR) Title 21, part 1271 aims at creating to create a unified registration and listing system for human cells, tissues, and cellular and tissue-based products (HCT/P's) and to establish donor-eligibility criteria, current GTP, and other procedures to "prevent the introduction, transmission, and spread of communicable diseases by HCT/P's" (www.FDA.gov). Cell products that are only minimally manipulated or subjected to homologous use without systemic effect are regulated solely by the Public Health Service (PHS) Act Section 361 and do not require a premarket review. Higher risk clinical trials that investigate ''more-than-minimally manipulated'' HCT/P's should follow the Investigational New Drug (IND) mechanism [3].

3 MSC Manufacturing

Regenerative medicine aims develop new innovative cell-based therapies. MSCs emerged as interesting candidates and are in various phases of clinical trials. The manufacturing process for MSCs is complex, involving the procurement of tissue as starting material, the isolation and most often the expansion of MSCs to yield clinically relevant cell doses [12, 13, 37, 84]. For cell-based products, sufficient numbers of vital and functional cells are required. Further, the product must not cause infections, allergies or malignancies. To verify this, numerous quality control steps need to be implemented within the manufacturing process, including microbiological tests (bacterial, viral, fungal, mycoplasma contamination as well as pyrogenicity testing), phenotyping to assess the degree of purity, functional testing (potency assays) as well as safety testing (tumorigenicity, gene expression profiles,
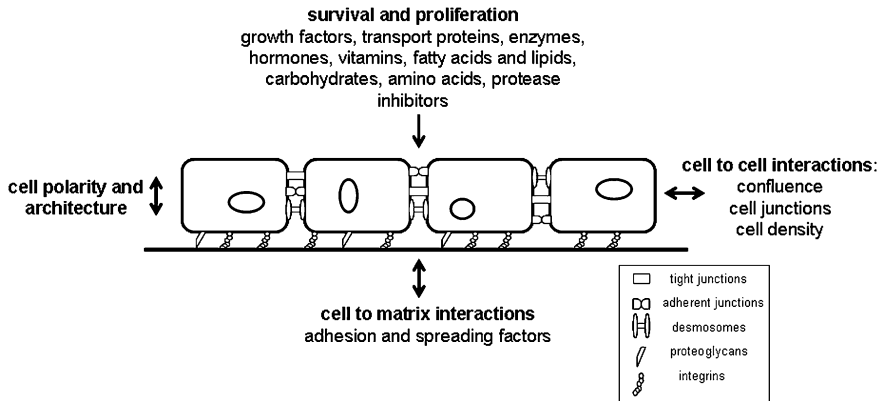
Fig. 1 Important factors affecting cell attachment, survival, proliferation and cell–cell/cell–matrix contacts provided by the culture conditions: basal medium and cell culture supplement

cytogenetics, etc.) [12, 13, 85]. Laboratories around the world developed various protocols, so far without standardization they even use reagents that are not indicated for clinical use, such as fetal bovine serum (FBS), which is critically rated by the European Medicines Agency [31]. Because of the likelihood of contamination, which was reported to be as high as 20–50 % for viruses in the late 1970s, it is not advisable to use these substances for cell-based medicinal products [101]. Because some treatments may require multiple cell applications and because MSCs internalize xenogenic proteins at high amounts, these substances can cause allergic reactions. Immunisation has already been demonstrated to compromise therapeutic success [41, 45, 83, 92, 94]. In light of these considerations, xenogenic-free culture conditions are desirable. Adaption of culture conditions, however, has to promote survival and proliferation and maintain the cell architecture and cell–cell interactions, as illustrated in Fig. 1. Thus, when changing the composition of the culture medium, which has numerous effects on cellular function, it is important to verify that all key cellular features are maintained. Differing culture conditions may isolate different MSC populations with different biological and functional features; for example, it may favour a specific differentiation pathway.

4 Towards Xenogenic-Free Culture Conditions for MSC Expansion

4.1 Fetal Bovine Serum

As indicated previously, the vast majority of protocols use FBS as a media supplement to maintain MSCs and support MSC proliferation. The routine use of FBS, however, poses clinical risks for patients. Besides the risks of transmissible

diseases and immunisation, the ability of processes to be standardized is poor. Serum in general is an ill-defined mixture of approximately 1,000 biological molecules, including growth factors, hormones, proteins, and vitamins, with a multitude of components still unknown [16]. Quality and concentrations of stimulatory and inhibitory growth factors vary between FBS lots; therefore, testing of various FBS lots is required [42].

The major functions of FBS are to provide:

  I. Growth factors and hormones
 II. Transport proteins
III. Attachment and spreading factors
IV. Amino acids and vitamins
  V. Fatty acids and lipids
 VI. Protease inhibitors
VII. Detoxification
VIII. (Colloid-) osmotic pressure

(see also Fig. 1 and Table 1) [16, 42, 96, 98]

Consequently, especially to finally achieve standardization, chemically defined media are required [98]. Because of the number of components serum provides, however, such media are hard to define. Although a number of serum-free, animal-derived component-free and chemically defined media are under development (Table 2), "breakthroughs" have not yet been achieved [64]. Information on the formulation of serum-free or chemically defined media as listed in Table 2 is often limited because of the proprietary development of companies. Plus, the majority of media are not yet approved as "GMP or clinical grade" but only as "research grade".

Of note, MSCs cultivated in FBS-replacement media need to be thoroughly investigated to verify that none of the biomarkers and especially none of the clinically relevant features are modified.

4.2 Serum-Free Cell Culture Media

Many FBS alternatives have been described, including newborn or adult bovine serum and serum from other species (horse, pig, goat, etc.). In contrast, serum-free culture media are not supplemented with serum; however, they may contain several proteins or bulk protein preparations, including animal or plant tissue extracts [71] such as bovine/porcine pituitary extract, chicken embryo extracts, ocular fluid, bovine milk fractions, or bovine colostrums [5, 34, 70].

Although serum-free media have better consistency, longer shelf-life and easier availability, they still pose the risk of transmitting xenogenic proteins, infections, immunization; in addition, they are still regarded as chemically undefined and thus poor in terms of standardization. A serum-free media interactive database has been developed by Brunner et al. [16], available at http://www.goodcellculture.com.

Table 1 Typical components of serum and basal medium [16, 42, 96]

Serum proteins	Albumin
Protease-inhibitors	Globulins (e.g. IgG)
Transport proteins	α1-Antitrypsin
Attachment and spreading factors	α2-Macroglobulin
Enzymes	Transcortin
	Transferrin
	α1-Lipoprotein
	β1-Lipoprotein
	Fibronectin
	Laminin
	Serum spreading factor
	Alanine aminotransferase (ALT/GPT)
	Alkaline phosphatase
	Aspartate aminotransferase (AST/GOT)
	Lactate dehydrogenase
	γ-Glutamyl transferase
Hormones	Corticosteroids
	Cortisol
	Glucagon
	Growth hormone
	Insulin
	Pancreatic glucagon
	Parathyroid hormone
	Pituitary glandotropic factors
	Prolactin
	Prostaglandins
	Testosterone
	Thyroid hormones
	Vasopressin
Growth factors and cytokines	Basic fibroblast growth factor (bFGF)
	Endothelial cell growth factor (ECGF)
	Epidermal growth factor (EGF)
	Glial growth factor (GGF)
	Insulin-like growth factor (IGF)
	Insulin-like growth factor-binding protein 2 (IGFBP2)
	Interferons
	Interleukins
	Nerve growth factor (NGF)
	Platelet factor (PF)
	Platelet-derived growth factor (PDGF)
	Prepro-insulin-like growth factor 1
	RANTES/CCL-5
	Transforming growth factor (TGF)
	Vascular endothelial growth factor (VEGF)
Soluble cell adhesion molecules	Soluble intercellular adhesion molecule (sICAM)
	Soluble vascular cell adhesion molecule (sVCAM)
Fatty acids and lipids	Free and protein-bound fatty acids
	Cholesterol
	Ethanolamine
	Phosphatidylethanolamine
	Phospholipids
	Triglycerides

Table 1 (continued)

Vitamins	Retinol/Retinoic acid (Vitamin A)
	Vitamin B-Group:
	Thiamine (Vitamin B_1)
	Riboflavin (Vitamin B_2)
	Niacinamide (Vitamin B_3)
	Pantothenic Acid
	(Vitamin B_5)
	Biotin (Vitamin B_7)
	Pyridoxal Phosphate
	(Vitamin B_6)
	Folic Acid (Vitamin B_9)
	Cobalamin (Vitamin B_{12})
	Ascorbic Acid (Vitamin C)
	α-Tocopherol (Vitamin E)
Carbohydrates	Fructose
	Galactose
	Glucose
	Glycolytic metabolites
	Mannose
	Ribose
Nonprotein nitrogens	Creatinine
	Polyamines
	Purines/Pyrimidines
	Urea
Essential amino acids	Arginine
	Cysteine
	Glutamine
	Histidine
	Interleucine
	Leucine
	Lysine
	Methionine
	Threonine
	Tryptophan
	Tyrosine
	Valine
Trace elements	Calcium (Ca)
	Chloride (Cl)
	Chromium (Cr)
	Cobalt (Co)
	Copper (Cu)
	Fluorine (F)
	Iodide (I)
	Iron (Fe)
	Magnesium (Mg)
	Manganese (Mn)
	Molybdenum (Mo)
	Nickel (Ni)
	Potassium (K)
	Selenium (Se)
	Sodium (Na)
	Tin (Sn)
	Vanadium (V)
	Zinc (Zn)

Table 2 Commercially available serum-free, animal-derived component-free and chemically defined media

Company	Product name	Main components	Animal/Human proteins	Applications	Publications
CellGenix™	CellGro® Preclinical-grade only GMP coming	Salts, sugars, amino acids, vitamins, buffers, phenol red (pH indicator) L-glutamine	Insulin (human recombinant, yeast-derived) Albumin (human-plasma-derived) Synthetic lipid containing chicken egg-derived lecithin (latter both licensed medicinal product for human use)	Serum-free expansion of MSC from umbilical cord matrix and blood	
Invitrogen	STEMPRO® MSC SFM Produced under cGMP	Low glucose, no glutamine, no HEPES	Contains components only of human origin (xeno-free)	Human MSC	[1, 21, 68]
StemRD	MesenGro®-MSC medium For research use only	Chemically defined, serum-free, xeno-free, no plate coating		For MSC from BM, adipose tissue and cord blood	
StemCell™ technologies	MesenCult®-XF Culture Kit (medium + attachment substrate) For research use only	Defined, xeno-free culture kit for human mesenchymal stem cells	Human apo-transferrin	Optimized for BM-MSC	[40]
ScienCell™	Mesenchymal Stem Cell Medium-serum free (MSCM-sf) For research use only	Essential and non-essential amino acids, vitamins, organic and inorganic compounds, hormones, growth factors, trace minerals; HEPES and bicarbonate buffered			

Table 2 (continued)

Company	Product name	Main components	Animal/Human proteins	Applications	Publications
VitroBiopharma	SC00B2: humanized complete medium optimized for growth/self-renewal of human MSCs; SC00B3: serum-free complete medium optimized for growth/self-renewal of MSCs		Allogeneic human serum		
R&D systems®	StemXVivo Serum-Free Human Mesenchymal Stem Cell Expansion Media. For research use only		Components derived from human plasma	MSC should be grown on extracellular matrix (ECM) protein coated plates	
Abcell-Bio	SPE-IV Intended for research	Synthetic iron carrier, rh-insulin, nucleosides, L-Glutamine, α-monothioglycerol, synthetic lipids, α-MEM Cytokines : rh-IGF-I, rh-b-FGF	Clinical grade human albumin	MSC from bone marrow, adipose tissue and cord blood Pre-adhesion molecules such as fibronectin and collagen required	Data available on www.abcell-bio.com

4.3 Xenogenic-Free Media

Numerous MSC studies now refer to "humanized" culture conditions. Humanized supplements tested to be FBS replacements include human serum, autologous or pooled allogeneic, cord blood serum and different platelet derivatives [96]. Human blood-derived products have been in clinical use for years. Their advantages include that they can be derived from healthy blood donors and tested according to blood banking standards for infectious and immunological parameters. Similar to FBS, human blood component-derived supplements include a variety of essential factors capable of promoting cell growth. However, also like FBS, the composition of human supplements is ill-defined.

4.3.1 Human Plasma

The number of publications referring to the use of plasma for MSC cultures is limited, likely because clotted serum (containing released factors of activated platelets) is much more effective than plasma in promoting cell proliferation [16]. Lin et al. reported sufficient proliferation of BM-MSCs in both 10% autologous plasma and serum, which was further enhanced by the addition of bFGF and EGF [63]. Trilineage differentiation capacity was preserved.

4.3.2 Human Autologous Serum

A few studies introduced human autologous serum (HAS) as a promising alternative to FBS. Stute et al. compared different doses of HAS in comparison to FBS. 10% HAS yielded comparable results to 10 % FBS regarding isolation and expansion and even enhanced osteogenic differentiation [93]. Other studies reported accelerated proliferation with significantly shorter population doubling times compared to FBS [52, 69, 72, 87]. The addition of bFGF further enhanced the proliferation rate compared with HAS alone [52, 72]. Importantly, in terms of genetic stability, HAS appeared to better maintain genetic and epigenetic stability than FBS [25].

To fulfill GMP criteria, HAS can be manufactured within a completely closed bag system [66]. Although HAS bears no additional immunological risk, it is not possible to standardize the quality. In addition, there is concern due to limited availability and possible autoantibodies [64].

4.3.3 Human Allogeneic Serum

Pooled human allogeneic serum (HS) can overcome the issue of variability and limited availability. Large donor pools can be better adjusted for a predefined quality as an "off-the shelf" product. Most commonly, serum from AB donors is

used to avoid presence of isoagglutinins, although MSCs appear not to express ABO blood group antigens [80].

In an early study from our group, pooled HS (individually pooled from at least five different healthy regular blood donors with blood group AB, tested for autoantibodies and fulfilling stringent blood donor eligibility criteria) was compared with FBS. HS supported an enhanced higher proliferation rate of adipose tissue-derived MSCs (ASCs) compared to FBS [54]. In some donors, lifespan exceeded that of FBS-cultivated cells, whereas in others similar cumulative population doublings were achieved within a shorter period of time [11]. The differentiation potential of MSCs and ASCs was maintained, along with immunoregulatory features [10, 54]. Importantly, in terms of safety, ASC cultivated in either FBS or HS underwent replicative senescence and failed to transform [11]. Cells cultured in HS appeared to be smaller with a more spindle-shaped morphology than those expanded in FBS. Importantly, the frequency of colony-forming-units (CFU-f) and the differentiation capacity into adipogenic and osteogenic lineages was similar for HS- and FBS-MSCs [54]. Thus, HS initially appears to be a feasible alternative to FBS that maintains the key features of MSCs. The smaller size, accelerated expansion and differing response to trypsinization, however, prompted us to compare the gene expression profiles of FBS- and HS-expanded ASCs. Within a microarray screening, 102 differential proteins were detected, with only 12 genes exhibiting a higher expression in HS [9]. Similar to previous data from Lange et al. and Shahdadfar et al., FBS-upregulated genes were mainly categorized by the gene clusters "differentiation/development" and "cell adhesion, extracellular matrix, and migration" [56, 87]. It was suggested that HS maintains MSCs in a premature/stemness stage. This finding corresponds to previously mentioned data by Dahl et al. that reported less genetic/epigenetic variation in HAS- than in FBS-supplemented MSC cultures [25]. Thus, the switch to FBS-containing culture conditions seems to particularly affect gene and protein expression of ASCs and possibly alters cellular features beyond those typically assessed when characterizing MSCs. Consequently, to ensure similar cellular qualities after changing bovine to "humanized" culture conditions, possible cellular changes (transcriptome, epigenome, proteome, secretome, etc.) need to be further addressed in comparability studies.

4.3.4 Allogenic Umbilical Cord Blood Serum

In recent years, human umbilical cord blood has been used as a source of hematopoietic stem cells and for transplantation procedures to treat various blood-related disorders. Allogenic umbilical cord blood serum (hUCBS) supports the growth, proliferation and differentiation of MSC [22, 49, 74, 75, 88, 97]. This is specifically related to the high expression level of cyclin D2, a cell cycle regulatory molecule [49, 74].

hUCBS combines a variety of growth factors (EGF, FGF, NGF, VEGF, PDGF, IGF, TGF, interleukins, interferons), human serum albumin (HSA), transferrin and metabolism promoting factors such as alpha-2 macroglobulin (protease inhibitor),

apolipoprotein B-100 precursor (cholesterol transporter), complement C3 precursor (regulation of VEGF production, G-protein-coupled receptor protein signaling pathway), complement C5 precursor (chemotaxis, positive regulation of chemokine production), and isoform 1 of complement factor H precursor (negative regulation of complement C3). The binding capacity of HSA to several small molecules is important for cell proliferation. Also, transferrin, as iron-transporting protein, directly regulates the cell cycle [91].

4.3.5 Human Platelet Derivatives

Platelet Lysate

Human platelets contain numerous factors to promote the growth of cells and cell lines [30]. Platelets play a major role in haemostasis. They promote blood clotting and wound healing after injury. After activation, platelets release a multifarious cytokine cocktail, including TGF-ß1 and 2, FGF, IGF-1, PDGF-AA, -AB and -BB, EGF, VEGF, platelet factor-4 (PF-4), attachment factors, protease inhibitors, mitogens, and coagulations factors [28, 65, 86]. Based on this cocktail, platelet-rich-plasma (PRP) and gel derived thereof has been used to augment bone regeneration [29], also in combination with MSCs [47]. These factors are also known to promote proliferation of MSCs [26].

Early studies evaluated the MSC growth-promoting effects of platelet growth factors in PRP released by calcium and thrombin stimulation [39, 50]. Both studies described accelerated expansion and migration, but differed with respect to osteogenic differentiation potential. The seminal work by Doucet et al. demonstrated that PRP-derived platelet lysate (PL) yields larger colonies and promotes MSC expansion and osteo-, adipo- and chondrogenic differentiation compared with FBS [28]. Similar to supplementation with HS, cells cultivated in platelet derivatives appear to have a more elongated, spindle-shaped form.

A number of studies then examined PL as a GMP-compliant substitute for FBS in MSC expansion [4, 6, 10, 24, 56, 81, 82]. Dirk Strunk and colleagues filed a US patent entitled "Plasma-free platelet lysate for use as a supplement in cell cultures and for the preparation of cell therapeutics" (Pub. No.: US 2009/0305401 A1; Dec 10, 2009). The patent comprises methods for preparing the PRP, removing the plasma, and lysing the platelets.

PL in general is derived by mechanical disruption of platelet concentrates by repeated freezing and thawing or chemical lysis of the membrane. Subsequent centrifugation steps separate the platelet debris from the supernatant, including all bioactive platelet factors present within the platelets. One advantage of platelet concentrate is that it can either be frozen immediately or used at the end of its shelf life (4–6 days after donation, depending on the current local regulations) [7, 33]. Thus those platelet concentrates not used for platelet transfusion can be allocated for PL manufacturing. This minimizes the decay of already donated units and consequently avoids an additional burden for blood donors.

The concentration of thrombocytes directly relates to the growth factor concentration. To evaluate the effect on MSC proliferation, different thrombocyte concentrations were tested: 1.5, 1.0, 0.75, and 0.5×10^9/mL (all 5% supplement in basal medium) [56]. Reduction of platelet concentration to less than 1.5×10^9/mL significantly impaired the pro-proliferative effect of higher concentrated PL. Centrifugation at 8,000 g compared to 900 g reduced the contamination with thrombocyte precipitates. PL increased not only size but also numbers of colonies; trilineage differentiation was conserved while immunomodulatory capacity was enhanced. The gene expression profile after microarray analyses highlighted a downregulation of several gene families including differentiation/development, cell adhesion/extracellular matrix–receptor interaction, TGF-ß signaling and thrombospondin-1 induced apoptosis. Gene clusters associated with cell cycle, DNA replication and purine metabolism were upregulated concomitant to the enhanced proliferation in PL.

A recent study compared GMP-grade PL obtained from pooled whole blood-derived buffy coats or from apheresis-derived platelet concentrates [33]. No significant differences regarding the cytokine content (bFGF, sCD40L, PDGF-AA, PDGF-AB/BB, sVCAM-1, sICAM-1, RANTES, TGF-ß1) were apparent, which was congruent with similar support of MSC proliferation. Titration from 2.5 to 20 % revealed 10 % to be the optimal concentration. To define those of relevance, specific growth factors were neutralized by antibodies. Interestingly, individual or combined inhibition of PDGF-BB, bFGF and also TGF-β1 reduced proliferation. A cocktail combining all three factors as recombinant proteins, however, failed to induce proliferation of BM-MSC even when adding the extracellular matrix molecules fibronectin and collagen I–III. Obviously, additional components are necessary to fully support MSC proliferation.

The supportive effect of some cytokines, often observed in FBS-supplemented cultures, may not necessarily hold true in "humanized" culture systems. Similar to our own yet-unpublished data, bFGF only supports expansion of FBS-supplemented BM-MSC cultures, but fails to do so in PL-supplemented systems [72].

Platelet Releasates

Whereas PL contains all bioactive platelet factors, the physiological repertoire of platelet factors is released after platelet activation. Accordingly, PL can contain aggregates of platelet membranes and membrane-associated platelet antigens, posing the threat of potential immunological reactions [54]. Thus platelet factors released by physiological stimuli might offer some advantages. Several processing steps have been shown to activate platelets, including thrombin, collagen, ADP/epinephrine and thrombin receptor activating peptide (TRAP) [54]. Interestingly, our own studies have shown that processed thrombin-activated platelet releasate in plasma (tPRP) and PL promote different proliferation rates of BM-MSCs and ASCs. Whereas PL promoted a significantly higher proliferation rate of BM-MSCs than tPRP [10], ASCs exhibited similar proliferative responses to PL and tPRP if at

all [10, 67]. Differential proteomics of PL and tPRP identified 20 differential proteins (Kinzebach et al. unpublished data). Identified proteins further denoted differences between BM-MSCs and ASCs: fibrinogen, for example, significantly supported the expansion of ASCs, and apolipoprotein A1 selectively reduced proliferation rate of BM-MSCs.

5 Pathogen Reduction Strategies

Although they have some advantages, human supplements still pose the risk of transferring infectious agents. Quarantine storage can in part overcome the risk of the diagnostic window. Quarantine storage allows a retest of the blood donor after a second donation after a time interval of at least 4 months. Only those units derived from donors verified to be negative for human infectious disease markers after the second donation are then pooled [33]. Alternatively, various pathogen-reduction strategies are under investigation to treat blood products [48]. A virally-inactivated PL was introduced by Shih et al. [90]. Here the PL was treated by solvent/detergent, then extracted by soybean oil and further purified by C18 chromatography and sterile filtration. Using a semiquantitative human cytokine antibody array cross reacted with some bovine proteins, the growth factor cocktail was compared. 22 cytokines were more highly concentrated in the virally-inactivated PL than in FBS, and only two cytokines (angiopoietin-2 and bFGF) were found at lower concentrations. As most often observed with BM-MSCs, PL induced massive proliferation compared to FBS. The typical MSC characteristics, phenotype, immune phenotype and differentiation were maintained in this virally-inactivated PL, indicating the feasibility of this approach.

6 Clinical Trials with MSCs Expanded in Human Supplements

A few studies listed in Table 3 already applied "humanized" culture conditions to expand MSCs for clinical trials. The study presented by von Bonin et al. evaluated BM-MSCs expanded for human PL in patients with refractory graft versus host disease (GvHD) [99]. In total, two out of 13 patients treated benefitted from the treatment. After a second dose, five out of 11 patients responded with a mitigation of their symptoms. A larger phase II study initiated by the European Society for Blood and Marrow Transplantation (EBMT) analyzed MSCs expanded in FBS; a complete response was demonstrated in 30 of 55 patients [58]. Furthermore, nine patients showed an improvement of GvHD symptoms. It was unclear whether or not these differences were related to the mode of MSC expansion, the other numerous variables inherent to the MSC production, the individual patient cohort, or possible differences in treatment regimens; further investigation is required. These examples, however, highlight that standardization of MSC manufacturing is

urgently needed to principally enable and finally facilitate comparison of clinical trial results. With the high numbers of variables still present, efficient clinical translation is significantly hampered.

7 Animal-Derived Component-free Media

Media lacking any components of animal or human origin are still not chemically defined. They may contain hydrolysates of bacteria, yeasts or plant extracts [98]. To the best of our knowledge, no such medium is currently propagated for MSC cultures.

8 Chemically Defined Media

As mentioned previously, human plasma, serum or platelet derivatives still contain ill-defined factors, which vary from donor to donor and can exert different biological effects, such as inhibition of proliferation and differentiation. In terms of standardization, these components cannot be regarded as ideal. Only a chemically defined medium can fulfil this criterion. It is obvious that the development of a chemically defined medium is complicated not only by the high number of components plasma, serum or platelet derivatives it contains but also by the combined action of these constituents. The efforts to define such media combining all physiologically relevant components are usually unsuccessful [64]. Within a chemically defined medium, all essential factors have to be present, including essential growth factors, attachment factors, nutrients, vitamins and transport proteins at precise concentrations. The challenge for MSCs is that the chemically defined medium has to support adhesion and subsequent proliferation of MSCs without altering the cell characteristics (Fig. 1). Most isolation protocols use the capability of serum-supplemented media (FBS, or human supplements) to selectively promote adhesion of MSC/CFU-f but not of other cell types. In combination with the selective support of proliferation (e.g., due to lack of other essential components/growth factors needed for endothelial cells) contaminating cells can be effectively eliminated within the primary or subsequent passage. Furthermore in vitro expansion ideally is enhanced compared with FBS to yield the high cell dose required for clinical application. This, however, has to be achieved without altering cellular characteristics or permitting the cells to enter an early senescence phase. It is essential that the clinically relevant features are maintained, including multilineage differentiation potential, stromal activity, immunomodulatory capacity, and trophic support.

In general, chemically defined media comprise basal media, such as Dulbecco's Modified Eagles Medium (DMEM) or Minimum essential medium alpha (α-MEM), into which the required bioactive factors are added. As indicated, the bioactive

Table 3 Clinical trials applying MSCs expanded in human supplements

Title	Condition	Source/Application	Status	Medium supplement
Treatment of steroid resistant GvHD by infusion of MSC ("MSC for GvHD")	Acute GvHD	Mismatched MSC	Phase I and II/ recruited	Human plasma and platelet lysate
Clinical trial based on the use of MSC from autologous bone marrow in patients with lumbar intervertebral degenerative disc disease	Intervertebral disc disease	Autologous bone marrow aspirate/ placement in the fusion bed during surgery	Phase I and II/ recruited	Platelet lysate
Treatment of maxillary bone cysts with autologous bone MSC (MSV-H) ("BIOMAX")	Maxillary cyst; Bone: loss of substance	Autologous bone marrow MSC	Phase I and II/ recruited	Autologous plasma
Clinical trials of regeneration for periodontal tissue	Adult periodontitis	Ex vivo cultured MSC and osteoblast-like cells differentiated from MSC and scaffold (platelet rich plasma, human thrombin and calcium chloride)	Phase I and II/ completed	Not specified
Treatment of refractory (acute or chronic) GvHD by the infusion of expanded in vitro allogeneic MSC	GvHD	Allogeneic bone marrow MSC	Phase I and II/ not yet recruiting	Autologous serum

http://clinicaltrials.gov: search 02/2012, search terms: MSC serum, human serum, platelet lysate

components of serum are diversified. Chemically defined formulations most often are composed of only approximately 10 essential components [98]. Subsequent to the challenge to identify those relevant for MSC culture, the interplay between concentration and combination of growth factors and synergistic effects (which can be either pro- or anti-proliferative) must be identified. The options to test are manifold. Accordingly, statistical approaches (e.g. fractional factorial design) are implemented to evaluate all possible combinations within the optimization process [32]. For example, although TGF-ß alone has no effect on MSC proliferation [21], the combination of TGF-ß with bFGF and PDGF results in a significant pro-proliferative effect [33, 68]. Gronthos and Simmons were among the first to describe the combined influence of various growth factors and other supplements in serum-deprived conditions. They found out that the combination of insulin, PDGF-BB, EGF with

dexamethasone and ascorbic acid can provide MSC expansion [38]. The invention by Cancedda and Dozine entitled "Serum-free medium for mesenchymal stem cells" (Patent US 7, 109, 032, B2, Sept 19, 2006) comprises the combination of basal minimum essential medium supplemented with albumin, transferrin, ß-mercaptoethanol, cholesterol, dexamethasone, leukemia inhibitory factor (LIF), stem cell factor (SCF), IGF-1, bFGF, PDGF-BB, EGF, ascorbic acid, sodium pentanone, biotin and selenium. It is demonstrated that selenium as a trace metal and biotin and pantothenate as vitamins support cell viability, whereas the combination of LIF, SCF and in particular IGF-1 promote cell proliferation.

In addition to the factors that promote proliferation, the selective adhesion of MSC is essential. Thus, attachment factors either must be added to the cell culture medium or the tissue culture vessel has to be coated prior to seeding the cells. Fibronectin is most commonly used, but gelatine, alginate or nanoscaffolds also can be used [46, 102].

In the meantime, a number of companies offer chemically defined media for MSC isolation and expansion, as listed in Table 2. Because of the rapidly evolving field, this table may not list all available media, but only those found within our search (2/2012).

9 Xenogenic-Free Cryopreservation Medium

Finally, we would like to briefly mention that the cryopreservation medium, in addition to the isolation and culture conditions of MSCs, should adhere to GMP and avoid undefined conditions. Zeisberger et al. compared the standard cryomedium [90 % FBS + 10 % DMSO (dimethyl sulfoxide)] with serum-/xenogenic-free chemically defined cryomedium IBMT with several DMSO concentrations (10, 5, 2, 0 %) [103]. Viability tests demonstrated that cryomedium IBMT + 5 % DMSO yielded no significant differences to the standard cryomedium.

10 Summary and Conclusion

For standardized MSCs manufactured on a routine basis chemically defined media approved for GMP and clinical use are regarded as the ultimate endpoint. Until this endpoint could be achieved, supplements derived from human blood products (serum or platelet lysate) emerged as reasonable transitional stages to replace FBS. As discussed, they can help to define those factors relevant for MSC isolation and expansion and, therefore optimize chemically defined media. It is already indicated which factors appear to be essential for which clinical features of MSCs (differentiation, immunomodulation, migration, etc.). Any change of culture conditions can have an impact on cellular qualities. Switching from FBS to human supplements induces measurable changes. Whether or not these changes impact cellular qualities

is, however, only poorly characterized. By reducing the amount of bioactive factors physiologically present in serum towards the few factors in a chemically defined medium, MSC qualities are further affected; and necessitates thorough investigation beyond the criteria demanded by the ISCT is required [27]. Of note, culture medium is only one building block in the complex structure of the GMP-compliant MSC manufacturing process. However, to provide successful MSC-based therapies, the establishment of standardized manufacturing protocols and quality control parameters and assays is of utmost importance. Based on the intensive research work in the translational field, it is expected that agreement on standardized protocols will enable comparable multicenter collaborative studies that assess feasibility, safety and efficacy. Identifying the impact of defined media components may also allow the creation of MSCs for specific therapeutic applications, similar to the differentiation media already applied to induce targeted differentiation of MSCs.

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Adipose-Derived Mesenchymal Stem Cells: Biology and Potential Applications

Danielle Minteer, Kacey G. Marra and J. Peter Rubin

Abstract Adipose tissue is derived from the mesoderm during embryonic development and is present in every mammalian species, located throughout the body. Adipose tissue serves as an endocrine organ, functioning to maintain energy metabolism through the storage of lipids. While two types of adipose tissue exist (brown and white), white adipose yields the commonly studied adipose-derived stem cells (ASCs). Adipose-derived stem cells provide a promising future in the field of tissue engineering and regenerative medicine. Due to their wide availability and ability to differentiate into other tissue types of the mesoderm—including bone, cartilage, muscle, and adipose—ASCs may serve a wide variety of applications. Adipose stem cells have been utilized in studies addressing osteoarthritis, diabetes mellitus, heart disease, and soft tissue regeneration and reconstruction after mastectomy and facial repair. Various delivery systems and scaffolds to incorporate adipose stem cells have also been established. Adipose stem cells have been studied in vitro and in vivo. Much information in vitro has been obtained on adipose stem cell potency and biology as a function of donor gender, body mass index, and anatomical location. Further in vitro studies have examined the various cell populations within the heterogeneous population within the stromal vascular fraction (SVF) from which ASCs are obtained. While many animal models are used to investigate adipose tissue, preclinical in vivo experiments are most widely conducted in the mouse model. Common analyses of animal studies utilizing ASCs include pre-labeling cells and immunostaining cells.

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Contents

1 Introduction: Adipose Tissue

1.1 Adipose Tissue Biology and Function

A knowledge and understanding of adipose-derived stem cell (ASC) biology is imperative to advance adipose-based therapies into clinical practice. Adipose tissue is present in all mammalian species and some nonmammalian species. It is located in subcutaneous tissues, the intraperitoneal compartment (visceral fat surrounding organs), and diffusely throughout the body as padding for vital structures [1]. Brown adipose tissue is highly functionally specialized and is abundant in mammalian infants, in whom it functions to maintain body heat. This mitochondria-rich brown fat is sparse in adults but can be found in the thorax and neck. This chapter focuses on white adipose tissue [2, 3]. Components of adipose tissue involve mostly mature lipid laden adipocytes and supporting tissue types: blood vessels, lymph nodes, nerves, and stromal-vascular cells.

Adipose tissue is derived from the mesoderm, along with other migratory cells, including the dermis, bone and cartilage, and the circulatory system (Fig. 1). Adipogenesis—the process of adipocyte maturation and subsequent fat tissue generation—involves proliferation of adipose stem cells (adipose precursor cells also known as preadipocytes) followed by the differentiation of the cells into mature adipocytes.

Proliferation and differentiation of ASCs is controlled via hormonal, neuronal, and paracrine pathways [1]. Specifically, thyroid hormones and glucocorticoids have been found to enhance the development of adipocytes in rats [4, 5] and porcine models, which are comparable to human fetuses during development [6–8].
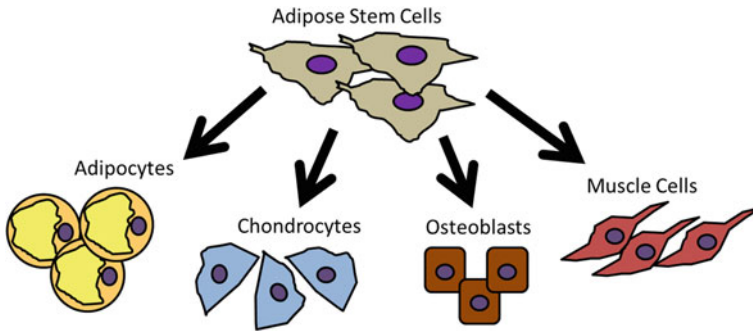
Fig. 1 Multilineage capacity of adipose stem cells. Derived from the mesenchymal germ line, adipose stem cells hold the multipotentiality to differentiate into adipocytes, chondrocytes, osteoblasts, or muscle cells with the addition of proper growth factor and under appropriate culture conditions

Furthermore, the glucocorticoid analogue dexamethasone is a widely accepted enhancer of preadipocyte recruitment and differentiation when incorporated with insulin [6]. The use of insulin and dexamethasone is currently being studied as a method of inducing regenerating fat tissue for reconstructive surgeries at the University of Pittsburgh [9]. The main paracrine signal that triggers adipocyte proliferation and differentiation is insulin-like growth factor-1 (IGF-1) [6].

Adipose tissue is critical for maintaining energy metabolism through storage of lipids—a task carried out by the mature adipocytes as a response to specific circulating hormones. Adipose function is multifactorial, encompassing endocrine functions, glucose metabolism, and lipid metabolism. These functional mechanisms overlap and interact with surrounding tissues and capillaries, as well as influence energy homeostasis throughout the entire organism [10]. Over 50 biochemical products are secreted by adipocytes, often with factors characteristic to different fat depots. The endocrine function of the adipocyte releases factors important to steroid metabolism, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemotactic protein (MCP-1), plasminogen activator inhibitor-1 (PAI-1), adiponectin, resistin, leptin, and angiotension. Several receptors are expressed on adipocytes for proteins involved in endocrine metabolism as well [11, 12].

Adipocytes store lipids in the form of triglycerides, which are presented to the adipocyte in the form of a glycerol molecule and three fatty acid chains. Once the free fatty acids are transported into the adipocyte, triglycerides are reformed and stored inside the lipid droplet. Hormone-sensitive lipase and lipoprotein lipase regulate triglyceride entry, storage, and release in the adipocytes. Knowledge of adipocyte biology and function, in addition to the key factors involved with endocrine, glucose, and lipid metabolism, is necessary for the study of obesity and the several chronic diseases associated with adipose tissue.
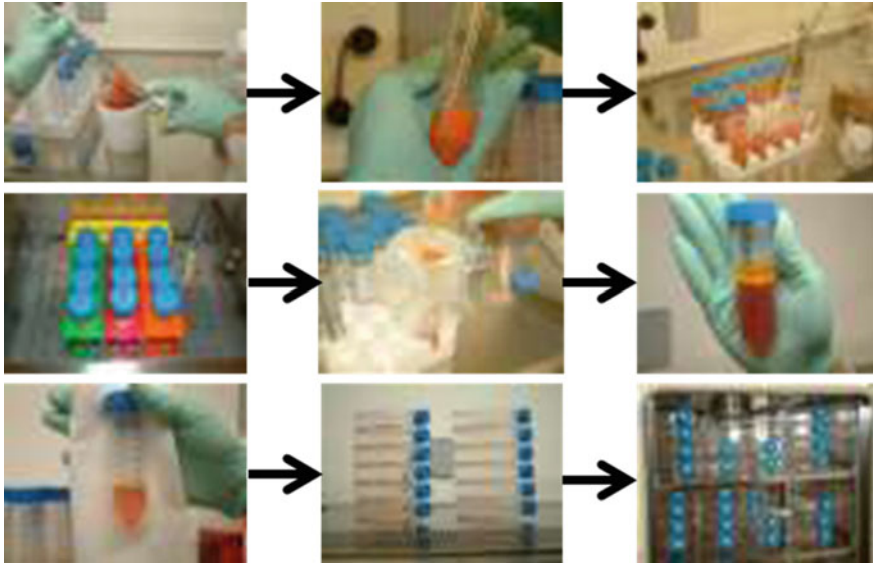
Fig. 2 ASC isolation. This schematic describes the isolation process of adipose-derived stem cells from discarded whole human fat tissue

1.2 Obesity and Ties to Chronic Diseases

Obesity remains an ever-growing pressing health issue in developed countries and is rapidly approaching epidemic status in the United States. According to the U.S. Centers for Disease Control and Prevention, approximately 33.8 % of American adults and 17 % of adolescents ages 2–19 years were obese in 2010 [13]. Using body mass index (BMI) (Eq. 1), "overweight" describes those with a calculated BMI between 25 and 30, whereas "obese" classifies those bearing a BMI greater than 30:

$$BMI = \frac{mass\ (kg)}{(height\ (m))^2} \tag{1}$$

It is known that obesity can induce chronic diseases, such as coronary artery disease, degenerative arthritis, type 2 diabetes mellitus, gall bladder disease, gout, hypertension, infertility, restrictive lung disease, stroke, and various types of cancers [14–18]. It is also known that diseases influenced by obesity are characterized by abdominal, visceral fat deposits, causing an "apple shape" (Fig. 2). Such an effect is known as the metabolic syndrome.

As previously mentioned, adipose tissue does function as an endocrine organ in addition to its glucose and lipid storage functions. As adipocytes increase in size and a person gains weight, several molecular and cellular changes occur and

ultimately influence whole-body metabolism. Horowitz et al. identified higher free fatty acid (FFA) and glycerol levels in obese women compared to lean women, suggesting a promotion of insulin resistance, which is the primary cause of type 2 diabetes [19, 20]. In addition to FFAs, several proinflammatory factors are secreted by adipose tissue, with an especially strong presence in those with obesity. TNF-α, IL-6, MCP-1, transforming growth factor-β (TGF-β), plasminogen activator inhibitor type I, tissue factor and factor VII are all elevated in obese individuals as compared to those with lean BMIs (<20) [14].

Adipose tissue is present throughout the mammalian body, whether under the skin to provide insulation or the surrounding organs to provide padding and protection. Although diseases such as type 2 diabetes and degenerative arthritis were traditionally thought to be diseases of the pancreas and heart, respectively, with relatively recent discoveries of fat additionally functioning as an endocrine organ, it is clear that expansion and proliferation of adipose tissue indeed affects whole-body homeostasis.

1.3 Clinical Applications: Overview

ASCs may be isolated from human adipose tissue and are known to have a high potential to differentiate into mature adipocytes and other tissue types along the mesenchyme lineage, including chondrocytes, osteoblasts, and skeletal and cardiac muscle [21–43]. Differentiation of ASCs into mature adipocytes has been studied in bioreactors (rotating wall [with and without microcarriers] and three-dimensional hollow fiber membrane-based) as methods of long-term adipocyte culture and high-throughput screening tools for drug discovery [44, 45]. However, one of the most promising and rapidly advancing clinical applications of ASCs lies in the field of clinical soft-tissue regeneration and reconstruction [46–54].

2 ASCs: An Established Cell Population in Biomedical Research

2.1 ASC Isolation and Culture Conditions

Standardized protocols to isolate adipose-derived stem cells from human adipose tissue are in place in many laboratories, such as the Adipose Stem Cell Center at the University of Pittsburgh [46, 47]. Discarded adipose tissue from elective surgeries is collected from the operating room. Whole fat tissue is chopped by hand with sterile scissors until finely minced. This mechanical processing step is not necessary for lipoaspirate, which is already in particulate form following harvest. To remove the fibrous collagen content of the tissue, a collagenase

solution is added to the minced fat and shaken at 37 °C until a fatty supernatant is clearly visible within the solution. Typically, after 25–30 min, a fatty layer rises and the tissue-collagenase solution should be centrifuged for 10 min at 1000 rpm at 4 °C. Next, the supernatant/fatty layer is aspirated and the pellet resuspended in an erythrocyte-lysing buffer to remove any red blood cells. Filtering and washing steps are performed with centrifugation at 1000 rpm, 4 °C, for 10 min. The resultant SVF pellet can be resuspended, cultured, and assessed for functional capacity [54]. ASCs will adhere to the surface of an untreated flask after approximately 6 h incubation at 37 °C and 5 % CO_2. Once ASCs have adhered to the culture flask surface, nonadherent populations are washed away with sterile phosphate-buffered solution and fresh culture media added to the flask. Essential points in the ASC isolation protocol are highlighted in Fig. 2.

A commonly used ASC expansion media consists of a DMEM and DMEM/F12 media combination, with 10 % serum, some form of antibiotic (typically penicillin/streptomycin), and typically a miniscule amount of dexamethasone, which prevents any differentiation into another mesenchymal lineage, such as osteoblasts.

2.2 Differentiation Potential of ASCs

"Pre-adipocytes" were first described in the 1970s, first in rat models [55, 56] and then isolated from human tissues in 1976 by Dardick et al. [57]. Isolated pre-adipocytes were used to study adipocyte biology in vitro and different anatomic locations and adipose depots became known to express different biological characteristics, such as adipocyte size and lipolytic potential [56]. In 2001, Zuk et al. first published the plasticity of differentiation of pre-adipocytes [21]. The stem cell features of "pre-adipocytes" became accepted and the term "adipose-derived stem cells" was given to encompass their characteristics of self-renewal, asymmetric division, and multipotency.

Over the past decade, several researchers have studied the ability of ASCs to differentiate both in vitro and in vivo. Although ASCs typically proliferate quite easily in culture, high concentrations of growth factors are necessary to induce lineage-specific differentiation. Differentiation of ASCs to other mesenchymal phenotypes has been well-established both in vitro and in vivo [21–34, 44–46, 58–60]. Differentiation of ASCs to cell lines of the ectodermal [23, 30, 35–37] and endodermal [38–42] germ layers has been studied, but evidence was often putative to show successful achievement of the desired phenotype.

It is imperative to note that both the SVF and ASC populations derived from tissues are not pure populations; numerous cell types exist within the SVF and ASC populations, confirmed by cell surface markers identified by flow cytometry [58, 59]. Zimmerlin et al. identified several similar cell surface markers in ASCs compared to bone marrow-derived stem cells; they are described in Table 1. Li et al. described four subpopulations of ASCs within the final stem cell pool cultured in vitro [61]. The first subpopulation is a CD31^{+}/34^{−} population classified as

Table 1 Cell surface markers of bone marrow-derived stem cells and adipose-derived stem cells, as derived from Zimmerman et al. [28]

	Bone marrow-derived stem cells	Adipose-derived stem cells
Positive	CD13, CD29, CD44, CD73, CD90, CD105, CD166, MHC class I, HLA-ABC	CD13, CD29, CD34, CD44, CD73, CD90, CD105, CD166, MHC class I, HLA-ABC
Negative	CD34, CD38, CD45 and for antigens involved in immunological signal transduction, such as HLA-DR, DP, DQ (MHC class II), CD80, CD86, CD40, and CD40L (CD154)	CD38, CD45, CD106, HLA-DR, DP, DQ (MHC class II), CD80, CD86, CD40, and CD40L (CD154)

"mature endothelial," having the endothelial marker of CD31 but lacking the progenitor marker of CD34. The second subpopulation is classified as "endothelial stem," with both $CD31^+/34^+$. A third subpopulation consisted of $CD34^+/31^-$ and is classified as the "adipose stem cell" group. The final subpopulation, as described by Li et al., represents a "pericyte group" and includes $CD146^+/90^+/31^-/34^-$. These cells reside adjacent to the endothelial cells, as demonstrated by immuno-staining [28]. As with bone marrow-derived stem cells, ASCs do not express MHC-II and do inhibit proliferation of activated peripheral blood mononuclear cells, suggesting a role for modulating the immune system in inflammatory disorders or allogeneic transplantation [58].

Studies identifying SVF and ASCs as heterogeneous populations emphasize the importance of defining subpopulation potential. Planat-Bénard et al. cultured the SVF from human adipose tissue in vitro and determined that a population spontaneously differentiated into cardiomyocytes without the addition of growth factor [22]. Cardiomyocytes were identified by morphology and confirmed by expression of cardiac-specific markers, immunohistochemistry straining, and ultrastructural analysis. The need for cardiomyocyte differentiation without the use of controversial, difficult-to-culture embryonic stem cells is in high demand in the field of cardiac regeneration. Planat-Benard et al. later used adipose-derived stem cells in the form of a "cell sheet" in rat [62] and nonhuman primate [63] chronic myocardial infarction models. The finding is certainly valuable in the field of cardiac engineering, and identifying a source of cardiomyocyte progenitors has been of great interest for therapeutic models targeted towards myocardial infarction.

2.3 Challenges in ASC Culture

A major challenge of using ASCs derived from human tissue lies in the variation between specimens harvested from different patients and also different subcutaneous depots in the same patient. Schipper et al. studied ASCs from five different subcutaneous adipose depots in 12 women with similar BMIs, split into three age

ranges: 25–30, 40–45, and 55–60 years [43]. The five subcutaneous depots studied were upper arm, medial thigh, trochanteric, superficial abdominal, and deep abdominal. The goal was to determine a group of cells most suitable for soft-tissue reconstruction applications. It was found that ASCs from younger patients proliferated at a faster rate than the ASCs isolated from older patients. Apoptosis of ASCs was found to be lowest in younger patients and from the superficial abdominal depot in all age ranges. Although lipolysis varied in both age and depot, the cells from the patients in the youngest age range had the highest activity in each adipose depot. The functional superiority of ASCs from younger patients and/or the superficial abdominal depot may have implications for tissue engineering applications.

The roles of gender and anatomical region on osteogenic differentiation of ASCs have also been studied in vitro [64]. ASCs isolated from the superficial and deep adipose layers of men and women were exposed to osteogenic differentiation medium for time points of 1, 2, and 4 weeks. Through alkaline phosphatase, alizarin red, and Masson trichrome staining, as well as enzyme-linked immunosorbent assay and Western blot analysis, the group was able to determine that no significant difference in the amount of osteogenic differentiation exists in both fat depots from women, whereas the superficial depot in men provided ASCs that differentiated sooner and more efficiently than ASCs from the deep fat depots. Furthermore, it was established that ASCs from men differentiated more effectively into osteoblasts than ASCs from women for all depots.

For large-scale culture experiments specifically examining adipogenesis, the murine 3T3-L1 cell line can be a useful model when consistency of cells is needed over time. These cells can be easily differentiated into adipocytes when stimulated with the proper conditions in vitro [65].

2.4 Pre-clinical Models

There are several animal models that are useful for the examination of adipose tissue engineering, and the mouse model has been the most widely examined [54, 66–72].

The effects of species, strain, gender, implant configuration, and implant location are all essential parameters when examining mesenchymal stem cells in preclinical studies [73]. When examining mesenchymal stem cells in small animals, there are two logical models for the researcher. One model uses a nude, or athymic, animal that will not reject human tissue or cells [74–76]. This model is ideal for examining the clinically relevant human cell. The second model involves injecting autogenous or syngeneic cells derived from the animal itself. For example, this model would entail isolating a population of cells from the strain of inbred mice, and injecting those cells into the mouse model. There are advantages and disadvantages of both of these approaches. Either option could result in a shortage of cells, depending on the mesenchymal stem cell source. A second

disadvantage of the nude mouse model is that it will not result in a similar response to that of the human response.

After the animals have been injected with stem cells, it can be challenging to identify and characterize the implanted cells. One method of quantification involves using pre-labeled cells. Cells can be labeled with cell membrane dyes, such as PKH26, or by using viral technology, such as green fluorescent protein (GFP) labels using lentiviral vectors. The expression of cell membrane dyes decreases with each cell doubling, and the dye could possibly leach into other cells. GFP-labeled cells, however, tend to remain stable throughout the lifetime of the animal. Finally, one can use immunostaining to identify the implanted cells. One challenge with immunostaining, however, is the potential for cross-reactivity of antibodies with both human and animal tissue.

3 Conclusion

Adipose tissue, developed in the mesoderm, is vital as insulation and protection to mammalian organisms; it also functions as an endocrine organ [6, 11, 12]. As the presence of obesity increases in developed countries, a continuous learning and understanding of the relationship between adipose tissue, inflammation, and metabolic diseases remains critical. The study of adipose-derived stem cells both in vitro and in vivo has provided plentiful information on the behavior and potency of the stem cells; cell culture has been thoroughly established, and it has provided several applications in soft tissue reconstruction [22–71]. Human ASCs in culture experience differences that are influenced by regional and anatomic locations, gender, and health; as a result, the 3T3-L1 cell line is commonly used [43, 64, 65]. The most common pre-clinical model of engineering ASCs involves the mouse model; GFP labeling of the cells also is a method of characterization.

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Potential for Osteogenic and Chondrogenic Differentiation of MSC

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Abstract The introduction of mesenchymal stem cells (MSC) into the field of tissue engineering for bone and cartilage repair is a promising development, since these cells can be expanded ex vivo to clinically relevant numbers and, after expansion, retain their ability to differentiate into different cell lineages. Mesenchymal stem cells isolated from various tissues have been intensively studied and characterized by many research groups. To obtain functionally active differentiated tissue, tissue engineered constructs are cultivated in vitro statically or dynamically in bioreactors under controlled conditions. These conditions include special cell culture media, addition of signalling molecules, various physical and chemical factors and the application of different mechanical stimuli. Oxygen concentration in the culture environment is also a significant factor which influences MSC proliferation, stemness and differentiation capacity. Knowledge of the different aspects which affect MSC differentiation in vivo and in vitro will help researchers to achieve directed cell fate without the addition of differentiation agents in concentrations above the physiological range.

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Keywords Chondrogenesis · Hypoxia · Mesenchymal stem cells · Mesenchymal stromal cells · Mechanical stimulation · Osteogenesis

Abbreviations

ALP	Alkaline phosphatase
AD	Adipose-derived
b-FGF	Basic fibroblast growth factor
BM	Bone marrow
BMPs	Bone morphogenic proteins
ESC	Embryonic stem cell
GAG	Glycosaminoglycan
GvHD	Graft-versus-host disease
h	Human
HA	Hyaluronic acid
HLA	Human leukocyte antigen
HUCPVC	Human umbilical cord perivascular cells
ISCT	International Society for Cellular Therapy
LIPUS	Low-intensity pulsed ultrasound
LLLI	Low-level light irradiation
MAPK	Mitogen-activated protein kinases
MMPs	Matrix metalloproteases
MSC	Mesenchymal stromal cell
PBL	Peripheral blood lymphocytes
PG	Proteoglycan
PLGA	Poly(L-lactide-co-glycolide)
PFF	Pulsating fluid flow
r	Rabbit
TGF-β	Transforming growth factor-beta
TE	Tissue engineering
UC	Umbilical cord
UCB	Umbilical cord blood
WJ	Wharton's jelly
3D	Three-dimensional

Contents

1 Introduction

The field of tissue engineering (TE) has attracted great attention over the last two decades, as traditional pharmaceutical products or artificial implants were not able provide effective/*sufficient* treatment options for several disorders and injuries, for example bone grafts substituting large defects after tumor resection or comminuted fractures, or cartilage replacement in degenerative joint disease.

One of the major principles of TE involves the use of biocompatible scaffolds, or of matrix that provides three-dimensional support for the growth of the cells that are seeded on it in vitro (ex vivo). To obtain specific functionally active tissue, the TE construct is cultivated in vitro statically or dynamically in bioreactors under controlled conditions, which include special cell culture media, addition of signalling molecules, various physical and chemical factors and additional mechanical stimuli (Fig. 1). The final construct can be implanted into the patient and represents an alternative to an artificial implant due to the fact that it has the capacity to adapt to changing demands of the body because of its biological potential for remodelling and growth. There is a great variety of three-dimensional biomaterials being used as scaffolds in TE. Generally, scaffolds can be divided into two groups: biological matrices (decellularized bone, blood vessel tissue, collagen, etc.) and synthetic scaffolds (hydroxyapatite, alginate, PLGA etc.).

One part of the TE construct consists of living cells that can be autologous or allogenic. The choice of the cells includes embryonic stem cells (ESC), adult stem cells (e.g. mesenchymal stem cells, MSC), or adult tissue-specific differentiated cells. Despite the wide potential of ESC, ethical restrictions present a big hurdle for utilisation of these cells. In contrast, adult tissue-specific cells cannot be easily isolated and expanded in vitro, a fact which makes it difficult to use these cells routinely in TE. Taking these considerations into account, MSC represent a valuable compromise as a cell source. On the one hand, there are very little or no ethical constraints to using these cells. On the other hand, even when isolated from adult tissues, they still retain their self-renewal and proliferational capacity, and they can be differentiated into various cell types in a controlled fashion. In this chapter, the potential of MSC of various origins for differentiation towards the osteogenic and chondrogenic lineage will be discussed. Factors that influence differentiation of MSC can be divided into chemical factors (e.g. media and nutrition components), physical
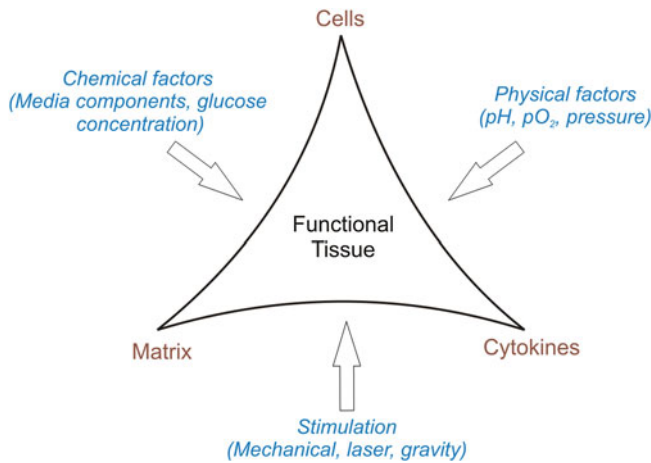
Fig. 1 Basic principles of tissue engineering and interfering parameters

Table 1 Sources of MSC and their differentiation capacities

Tissue	Differential potential	Reference
Bone marrow	Adipogenic, chondrogenic, osteogenic, myogenic, neuronal	[5]
Adipose tissue	Adipogenic, chondrogenic, osteogenic, myogenic	[5, 6]
Cartilage	Adipogenic, chondrogenic, osteogenic	[7]
Dermis	Adipogenic, chondrogenic, osteogenic, myogenic	[5]
Dental pulp	Adipogenic, chondrogenic, osteogenic, myogenic, neuronal	[8]
Breast milk	Adipogenic, chondrogenic, osteogenic	[9]
Blood	Adipogenic, osteogenic, osteoclastic, fibroblastic	[5, 10]
Umbillical cord blood	Adipogenic, chondrogenic, osteogenic, neuronal, epithelial, hepatogenic, myogenic	[11–14]
Urine	Urothelial, myogenic	[15]
Wharton's jelly	Adipogenic, chondrogenic, osteogenic, myogenic, neuronal, endothelial, hepatogenic, pancreagenic	[16–18]
Placenta/chorion	Chondrogenic, osteogenic, myogenic, neuronal	[16]
Placenta/amnion	Adipogenic, chondrogenic, osteogenic, myogenic, endothelial	[16]

factors (e.g. atmospheric pressure or oxygen concentration) and application of additional stimulation (e.g. mechanical stimuli and laser irradiation) (Fig. 1).

2 MSC Sources for Cartilage and Bone Tissue Engineering

Since their first isolation in 1970 from guinea pig bone marrow aspirates [1], MSC have been found in almost all postnatal tissues (Table 1). In vivo, these cells can participate in tissue regeneration via differentiation or paracrine rescue function. MSC isolated from different sources must fulfill the minimal criteria established

by the International Society for Cellular Therapy (ISCT): (i) adherence to plastic under standard culture conditions, (ii) differentiation towards osteogenic, chondrogenic and adipogenic lineage, and (iii) specific surface antigen expression [2]. However, despite the commonly accepted criteria, there are no universal specific MSC markers, and isolated cells usually represent a heterogeneous cell population [3]. MSC from different sources can be successfully expanded in vitro and optimal cultivation protocols have been developed for different types of MSC [4]. Besides the mesenchymal differentiation potential, most of the cells also demonstrate translineage differentiation capacity, e.g. towards neuronal cells [5].

3 Standard Media Supplements for Osteogenic and Chondrogenic Differentiation

The standard reproducible protocol for inducing osteogenic differentiation was developed for bone-marrow-derived MSC and included 16 days of differentiation of confluent cell culture in 3–4-passage in medium, supplemented with dexamethasone, ascorbic acid and beta-glycerophosphate [19]. Dexamethasone is a synthetic glucocorticoid that was shown to induce the differentiation of MSC towards osteogenic, adipogenic and chondrogenic lineages (depending on the concentration applied) in combination with other factors. Although the exact mechanism of dexamethasone-induced differentiation is not clear, it was shown that it augments the responsiveness of progenitor cells to other differentiation reagents but that it does not define the lineage. Dexamethasone also causes apoptosis of cell populations with poor differentiation capacity [20].

Another important supplement of osteogenic, adipogenic and chondrogenic culture media is ascorbic acid, which has an antioxidant function, and is also an essential cofactor for prolyl lysil hydrolase, a key enzyme of collagen biosynthesis [21]. 1,2,5-Dihydroxyvitamin D_3 (the active form of vitamin D_3) is another component commonly used to enhance osteogenic differentiation.

Major growth factors that regulate or enhance osteogenic differentiation of progenitor cells during prenatal bone development in vivo are bone morphogenetic proteins (BMPs). They also play an important role in fracture repair in adult organisms. Multiple BMPs are produced endogenously by bone marrow MSC and their level is dependent on the degree of osteogenic differentiation [22, 23]. It was shown that the central regulator of the network of BMPs is BMP-2.

The promotion effect of BMP-2 and vitamin D_3 on osteogenic differentiation of adipose-derived (AD)-MSC was shown by Song and colleagues, who demonstrated an increased level of alkaline phosphatase (ALP) expression, and, subsequently, mineralization of the treated cells [24]. It was also shown that BMP-2 and vitamin D_3 work synergistically. For umbilical cord blood (UCB)-MSC, it was revealed that dexamethasone seems to be a leading osteoconductive factor, since successful matrix mineralization could be achieved with enhanced concentrations of dexamethasone (10^{-7} M) even in the absence of BMP-2 [25].

A standard protocol for chondrogenesis was also first established for bone marrow (BM)-MSC [26, 27] and, besides the above-mentioned ascorbic acid and dexamethasone, contains transforming growth factor-beta (TGF-β) and proline. TGF-β superfamily members were shown to be a key requirement for chondrogenesis of MSC [28, 29]. TGF-β induces chondrogenic differentiation of MSC via different pathways, including the Smad [30, 31], extracellular signal-regulated kinase1/2 and c-Jun N-terminal kinase pathways [32].

4 Chondrogenic and Osteogenic Potential of MSC In Vitro

MSC from different sources display different potentials to differentiate towards certain cell lineages. Often the potential is dependent on the culture conditions and protocols. Cells can be differentiated in cell pellet 3D cultures or as monolayers. Culture time also varies. AD-MSC were found to express genes and proteins for cartilage-specific molecules, including type II collagen and aggrecan, but lacked expression of hypertrophic chondrocyte markers such as type X collagen [33–35]. BM-MSC were shown to have a chondrogenic differentiation capacity independent of donor age and osteoarthritis [36]. Payne and colleagues, however, reported that only those BM-MSC from female donors retain their chondrogenic potential with age [37].

When compared to BM-MSC, AD-MSC demonstrated statistically weaker chondrogenic potential in terms of matrix formation and cell morphology [38]. These findings were supported by another group testing the chondrogenic potential of BM-MSC and AD-MSC from the same donors. Here again, despite elevated specific gene expression, histological, immunohistochemical and glycosaminoglycan (GAG) assays clearly showed that collagen II and proteoglycans (PG) were synthesized only in the BM-MSC [39]. When cultivated on hyaluronic acid (HA) scaffolds, AD-MSC were less efficient with regard to chondrogenesis than BM-MSC [40]. Some authors suggested the use of higher concentrations of specific growth factors to achieve desirable chondrogenic differentiation of AD-MSC [41]. No difference in the chondrogenic potential between AD-MSC and BM-MSC was revealed in the work of Kern and colleagues [42].

Unlike AD-MSC, UC-MSC have a very high chondrogenic potential. When seeded on polyglycolic acid 3D scaffolds, UC-MSC produced three times more collagen as BM-MSC [43]. In cell pellet culture, UC-MSC had a comparable differentiation capacity to BM-MSC in terms of GAG level and Alcian Blue staining intensity with a pellet size that was bigger in UC-MSC culture than in BM-MSC [44]. Contradictory results were obtained by Hildner et al. who demonstrated higher chondrogenic potential of AD-MSC than UC-MSC [45]. Nevertheless, in the case of UC-MSC the problem of donor-age dependency can be neglected since all cord tissues are obtained from nearly the same full-term gestation deliveries.

Regarding osteogenic potential, BM-MSC display a very high capacity to differentiate into osteocytes. As in chondrogenic differentiation, direct comparison

Table 2 Influence of oxygen concentration on proliferation and differentiation capacity of MSC

Oxygen concentration (%)	Type of cells	Observed effect	Reference
≤ 1	rBM-MSC	Increased proliferation, induced ALP activity and production of collagen I/III	[54]
≤ 1	hBM-MSC	Down-regulation of several osteoblastic markers	[55]
≤ 1	hBM-MSC	Decreased osteogenesis via suppression of RUNX2	[56]
1	hBM-MSC	Decreased proliferation and differentiation	[57]
2	hBM-MSC	Prolonged stemness, increased proliferation	[58]
2	hWJ-MSC	Increased proliferation and increased expression of mesodermal and endothelial markers	[59]
2	hAD-MSC	Decreased chondrogenesis and osteogenesis	[60]
3	hBM-MSC	Decreased osteogenesis	[61]
5	rBM-MSC	Increased proliferation, ALP activity and osteogenesis in vivo and in vitro	[62]
5	hAD-MSC	Increased proliferation, collagen II synthesis and chondrogenesis	[63]
5	hAD-MSC	Increased chondrogenesis, decreased osteogenesis	[64]

showed a weaker osteogenic potential of AD-MSC than BM-MSC—that is, less ALP staining and lower degree of matrix mineralization [38]. In another study, AD-MSC and BM-MSC seeded on scaffolds displayed a similar degree of differentiation [46]. Two other groups also did not observe a difference in chondrogenic and osteogenic differentiation capacities between AD-MSC, BM-MSC and UCB-MSC [42, 47].

5 Influence of Oxygen Concentration on Differentiation Capacity of MSC

Oxygen is a key substrate in cellular metabolism, and also an important signalling molecule. Numerous working groups have been studying the influence of oxygen concentration on differentiation capacity (Table 2). Commonly, cell and tissue cultivation employs an ambient oxygen concentration of 21% O_2. In vivo, however, most cells of the body are not exposed to such high oxygen concentrations. Cartilage is an avascular tissue and thus resides in a microenvironment with reduced oxygen tension; oxygen concentration in articular cartilage is between 1 and 5% O_2 [48]. Oxygen measurements revealed that bone marrow is also hypoxic, with some regions as low as 1–7% O_2 [49, 50]. Adipose tissue is more vascularized, with oxygen concentrations measured to be between 10 and 15% O_2 [51]. Oxygen concentrations in this range are called "physiological hypoxia" or "in situ normoxia" [52]. On the other hand, avascularized TE constructs directly

after transplantation can suffer from limitation in oxygen and nutrition supply and it is important to understand the behaviour of the cells under hypoxic conditions. Although the results of different studies are often dissimilar, it can be concluded that hypoxia increases cell proliferation [53], helps cells to retain their stemness longer, increases chondrogenic differentiation, but inhibits differentiation towards osteogenic lineage. It is evident that optimal oxygen concentrations are still to be found for certain cell types and differentiation directions.

Hirao and colleagues showed that a hypoxic microenvironment promotes a chondrogenic rather than an osteogenic phenotype [65]. Other researchers showed similar results, concluding that hypoxic conditions promote the chondrogenesis of MSC [66–69]. Direct comparison of dynamic compression and low oxygen tension revealed that hypoxia is a more potent pro-chondrogenic stimulus than mechanical stimulation [70]. Moreover, expansion of BM-MSC under low oxygen tension (5%) enhanced their subsequent osteogenesis [69].

Cultivation under low oxygen concentrations had the same effect on AD-MSC, namely stronger chondrogenesis and inhibited osteogenesis [64]. Merceron and colleagues concluded that TE constructs for bone repair should contain a capillary network or angiogenic factors along with sufficient porosity of scaffolds.

Undoubtedly, oxygen concentration is a very important cultivation parameter. By manipulating oxygen concentration during cell expansion or differentiation, one can steer the cells' fate in the desired direction without adding supplementary chemical or biological factors.

6 Mechanical Stimulation

As early as in 1892, Julius Wolff, a German surgeon and anatomist, published the theory that bone formation is dependent on the mechanical forces applied to it, in his book "Das Gesetz der Transformation der Knochen" [71]. This theory was named later Wolff's law. Mechanical loading of bone cells is transmitted via fluid-flow or mechanical strain-induced mechanisms [72, 73]. In cartilage, hydrostatic pressure was shown to be a pivotal mechanical stimulator of matrix synthesis [74]. MSC are mechanosensitive and actively respond to mechanical stimuli, differentiating depending on the applied force into osteocytes or chondrocytes [75, 76]. Although the exact mechanism by which the mechanical force is translated by the cells into biological signals remains unclear, integrins and ion channels have been shown to be involved in mechanotransduction as mechanoreceptors [77]. Mechanical stimuli regulate the expression of matrix metalloproteases (MMPs), which cleave substrate proteins to release differentiation factors, such as TGF-β [77]. Other components of mechanotransduction are nitric oxide and prostaglandin E_2, which regulate the expression of different transcription factors, e.g. RUNX2 [77]. Several theoretical models have been developed to predict the effect of mechanical stimuli on the differentiation of precursor cells [76]. The necessity of applying controlled mechanical stimuli that simulate the environment in vivo to direct cell differentiation has resulted in the development of different types of bioreactors.

6.1 Fluid Flow

In-vivo mechanical loading of bone plays an important role in bone resorption and formation. One of the mechanical forces applied to the bone cells in vivo is interstitial fluid movement through lacunae. The influence of fluid flow on MSC differentiation has been intensively studied both in monolayers and within a 3D scaffold. Monolayer cell culture experiments are usually performed in different types of flow chambers, where fluid passes over the surface of the cell layer. The low magnitude of shear stress (0.3 dyn/cm^2) in the presence of differentiation factors induces osteogenesis of BM-MSC in terms of intracellular calcium immobilization and increased osteoponin and osteocalcin mRNA levels [78, 79]. It was also suggested that under flow stress an immature non-osteogenic subset of cells that is unable to respond with appropriate mechanotransduction and osteogenic specification proceeds to apoptosis and/or detachment [79]. MSC from dental tissue were responsive to pulsating fluid flow (PFF) (0.7 ± 0.3 Pa, 5 Hz) [80]. AD-MSC also responded to PFF in the presence of vitamin 1,25-dihydroxyvitamin D$_3$ and demonstrated an increased production of nitric oxide and cyclooxygenase-2 gene expression [81]. Glossop and colleagues studied the gene expression profiles of BM-MSC under different magnitudes and durations of fluid-flow-induced shear stress. They revealed dissimilar responses of MAPK signalling pathways to different profiles of shear stress, where MAP3K8 appeared to be an important mediator of intracellular mechanotransduction in human MSC [82].

Cells seeded on scaffolds also demonstrate increased osteogenic differentiation in dynamic (fluid-flow) cultures. BM-MSC on silicate-substituted tricalcium phosphate scaffolds had more pronounced expression of several bone markers, e.g. ALP, osteopontin, RUNX2, bone sialoprotein II, and BMP-2 [83]. In rotating bioreactors with dynamic culture conditions, osteoblasts seeded on poly (lactide-*co*-glycolide) scaffolds had enhanced calcium deposition, ALP activity and osteocalcin and osteoponin expression level in response to fluid flow [84]. Cultivation of different types of cells on Sponceram$^®$ matrix in rotating bed bioreactors also demonstrated encouraging results in regard to osteogenic differentiation [85, 86].

6.2 Mechanical Strain

Another type of stimuli that can be applied to MSC to enhance their osteogenic differentiation is mechanical strain. Mechanical strain has been shown to promote osteogenesis of BM-MSC in vitro, verified by the upregulation of osteogenic marker proteins like alkaline phosphatase, osteocalcin, osteopontin and type I collagen [87–90]. There are several approaches to application of mechanical strain to the cells, including circular membranes, longitudinal stress and 4-point bending [91]. One of the examples of longitudinal stress application is flexible silicone dishes, connected to the straining device with an eccentric motor (Fig. 2). Here,
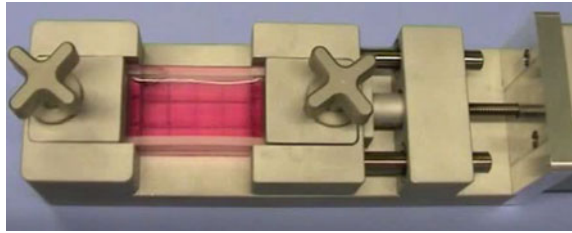
longitudinal stress applied cyclically to AD-MSC caused increased osteogenesis when a gradually increasing strain scheme was used (starting with short-term strain and followed by consecutively lengthened strain periods) [92].

6.3 Dynamic Compression

Dynamic compressive loading is the major mechanical force to which chondrocytes are exposed in vivo. Chondrocytes respond to physiological levels of dynamic compression with enhanced cartilage-specific micromolecule biosynthesis [93–96]. Kisiday and colleagues demonstrated stimulation of proteoglycan synthesis by MSC in response to dynamic compression [97]. It was shown that MSC have the fundamental ability to distinguish between different types of physical forces by regulating distinct gene expression patterns; e.g. dynamic tension was found to regulate both fibroblastic- and osteogenic-associated genes while dynamic compression up-regulated genes associated with chondrogenesis [98]. It was also discovered that mechanical stimulation of MSC over compression promotes chondrogenesis via the TGF-β pathway by up-regulating TGF-β gene expression and protein synthesis [99]. Although dynamic compression increased GAG synthesis in MSC [100], biochemical stimulation via supplementation of cell culture media with TGF-β3 was shown to be more effective for chondrogenesis of BM-MSC than dynamic compression [101]. Oxygen tension was also shown to be a more potent inducer of chondrogenesis than dynamic compressive loading [70]. It should be noticed, however, that chondrogenic induction of MSC in vitro is performed with supraphysiological concentrations of TGF-β. In vivo, where no exogenous TGF-β is present, mechanical microenvironment and local oxygen tension play a more important role in determination of the implanted cell fate.

6.4 Laser Irradiation, Ultrasound and Microgravity

Low-level light irradiation (LLLI) uses low-level lasers or light-emitting diodes to alter cellular function. It has been shown that LLLI stimulates cell proliferation and increases growth-factor synthesis and angiogenesis [102, 103]. Although the exact

mechanism of the mitogenic effect of LLLI is unknown, several theories have been proposed [104]. For MSC, it has been demonstrated that LLLI increases cellular viability, proliferation, and myogenic and osteogenic differentiation [105–107]. cDNA microarray analysis revealed that after LLLI the expression levels of various genes involved in cell proliferation, apoptosis and the cell cycle were affected [108].

Ultrasound is a widely used medical tool for diagnostic, operations and therapy. Therapeutic application of ultrasound is usually aimed at mild heating of living tissues, while surgical use involves cataract ablation and calculi fragmentation. The first clinical use of ultrasound for fracture healing was as early as 1953 [109]. Later, numerous clinical and animal studies confirmed the stimulatory effect of ultrasound on bone healing (reviewed in [110]). In MSC, low-intensity pulsed ultrasound (LIPUS) was shown to have a positive effect on osteogenic differentiation [111].

If cultivated on 3D scaffolds (microcarriers) in rotating-wall vessel bioreactors or spinner flasks, cultured cells are exposed to a simulated microgravity environment [112]. Microgravity-induced bone lose has been described as a response to absence of gravity and, consequently, of mechanical loading during space flight [113, 114]. MSC cultivated under microgravity conditions were demonstrated to decrease their osteogenic differentiation potential, while the percentage of cells committed to the adipogenic lineage was increased [115]. Gene expression analysis supported these findings, showing a significant decrease in osteogenic and chondrogenic gene expression and increase in adipogenic expression profile [116]. The development of new bioreactor systems where MSC are cultivated on microcarriers or free-floating 3D scaffolds should therefore consider the fact that in certain conditions cells can be exposed to lower gravity which, in turn, may affect their differentiation capacity.

MSC derived from different tissues represent an outstandingly interesting resource for therapeutic use in humans. Many clinical trials are currently being carried out with some legitimate positive expectations and promising results. The prerequisite is the provision of relevant cell numbers generated under well-controlled and reproducible conditions, ensuring safe expansion and guided differentiation of MSC. In this chapter, we have summarized some of the relevant work within this field.

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Potential for Neural Differentiation of Mesenchymal Stem Cells

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Abstract Adult human stem cells have gained progressive interest as a promising source of autologous cells to be used as therapeutic vehicles. Particularly, mesenchymal stem cells (MSCs) represent a great tool in regenerative medicine because of their ability to differentiate into a variety of specialized cells. Among adult tissues in which MSCs are resident, adipose tissue has shown clear advantages over other sources of MSCs (ease of surgical access, availability, and isolation), making adipose tissue the ideal large-scale source for research on clinical applications. Stem cells derived from the adipose tissue (adipose-derived stem cells = ADSCs) possess a great and unique regenerative potential: they are self-renewing and can differentiate along several mesenchymal tissue lineages (adipocytes, osteoblasts, myocytes, chondrocytes, endothelial cells, and cardiomyocytes), among which neuronal-like cells gained particular interest. In view of the promising clinical applications in tissue regeneration, research has been conducted towards the creation of a successful protocol for achieving cells with a well-defined neural phenotype from adipose tissue. The promising results obtained open new scenarios for innovative approaches for a cell-based treatment of neurological degenerative disorders.

Keywords MSC · Adipose derived stem cells · Neuronal commitment · Clinical application

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Abbreviations

ADSCs	Adipose-derived stem cells
ALP	Alkaline phosphatase
ATMPs	Advanced therapy medicinal products
BDNF	Brain-derived neurotrophic factor
BM	Bone marrow
BMI	Body mass index
BMP	Bone morphogenetic protein
CNS	Central nervous system
DMEM	Dulbecco's modified Eagle's medium
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GFAP	Glial fibrillar acidic protein
GMP	Good manufacturing practice
HBSS	Hank's balanced salts solution
HGF	Hepatocyte growth factor
IF	Intermediate filament
IGF	Insulin growth factor
IHC	Intracerebral hemorrhage
MAs	Multicellular aggregates
MCAO	Middle cerebral artery occlusion
MSCs	Mesenchymal stem cells
NGF	Nerve growth factor
NT	Neurotrophin
P	Passage
PLA	Processed lipoaspirate
PNS	Peripheral nervous systems
ROS	Reactive oxygen species
rpm	Revolution per minute
SCI	Spinal cord injury
SCs	Schwann cells
SVF	Stromal-vascular fraction
TBI	Traumatic brain injury
TGF	Transforming growth factor
TNF	Tumor necrosis factor
UCB	Umbilical cord blood

Contents

1 Mesenchymal Stem Cells

A stem cell is defined as a cell that has the ability to continuously divide to either replicate itself (self-renewing), or produce specialized cells that can differentiate into various other types of cells or tissues (multilineage differentiation). The microenvironment in which stem cells reside is called a stem cell niche and is composed of heterogeneous cell types, extracellular matrix (ECM), and soluble factors to support the maintenance and self-renewal of the stem cells [104]. Stem cells can be generally classified as embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs derive from the early mammalian embryo at the blastocyst stage and have the capability to give rise to all kinds of cells. Thus, ESCs are considered pluripotent. On the contrary, ASCs are just multipotent because their differentiation potential is restricted to certain cell lineages. ASCs reside in several and perhaps most organs and tissues that have already developed. For this reason, ASCs are also referred to as post-natal stem cells.

Mesenchymal stem cells (MSCs) are ASCs with mesodermal and neuroecto-dermal origin [110]. MSCs are able to differentiate into cells of mesodermal origin such as adipocytes, chondrocytes, or osteocytes, but they can also give rise to representative lineages of the three embryonic layers. For instance, it is well known that MSCs possess an extended degree of plasticity compared to other ASCs populations, including the ability to differentiate in vitro into nonmeso-dermal cell types such as neurons and astrocytes. Bone marrow (BM) MSCs are currently considered the gold standard by which newly discovered sources of MSCs are compared on the basis of renewal and multipotency. BM-MSCs are typically isolated from the iliac crest, but they have also been found in other BM cavities such as vertebrae bodies [2].

Apart from BM, MSCs can be isolated from trabecular bone [90], periosteum [23], synovial membrane [26], skeletal muscle [27], skin [11], pericytes [31], peripheral blood [86], deciduous teeth [68], periodontal ligament [85], and the term placenta (amniotic and chorionic membranes and the connective tissue of the umbilical cord named Wharton's jelly; [89]. Although the stem cell populations derived from these sources are valuable, common problems include low numbers of harvested cells and limited amounts of harvested tissues [16, 37, 73]. For these reasons, many researchers began to investigate alternative tissues for more abundant and accessible sources of MSCs with least invasive collection procedures.

2 MSCs from Adipose Tissue: ADSCs

Recent studies have shown that subcutaneous adipose tissue provides a clear advantage over other MSCs sources due to the ease with which adipose tissue can be accessed (under local anesthesia and with minimum patient discomfort) as well as to the ease of isolating stem cells from the harvested tissue [18, 70].

Stem cell frequency is significantly higher in adipose tissue than in BM: The yield of adipose-derived stem cells (ADSCs) is approximately 5,000 fibroblast colony-forming units (CFU-F) per gram of adipose tissue, compared to BM, which contains approximately 100–1,000 CFU-F per ml of BM [81]. Moreover, maintenance of the proliferating ability in culture seems to be superior in ADSCs compared with BM-MSCs [75].

ADSCs are isolated through an initial enzymatic digestion of the harvested adipose tissue, which yields a mixture of stromal and vascular cells (preadipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, resident monocytes/macrophages, lymphocytes, and ADSCs; [95], referred to as the stromal-vascular fraction (SVF) [100]. SVF is a rich source of pluripotent ADSCs [49, 111], which were first identified by Zuk and named processed lipoaspirate (PLA) cells [111, 112]. The selection of ADSCs out of SVF is based on their physical adherence to plastic tissue culture dishes.

When it comes to the nomenclature used to describe ADSCs, there is no consensus: they are variously termed preadipocytes, stromal cells, PLA cells, multipotent adipose-derived stem cells, or ADSCs. However, at a consensus conference of the International Fat Applied Technology Society, the term ''adipose-derived stem cells'' (ADSCs) was recommended for consistency among research groups [38].

Morphologically, ADSCs are fibroblastlike cells and preserve their shape after in vitro expansion [6, 107, 111]. Average doubling time of tissue-cultured ADSCs is between three [111] to 5 days [42]. They are MSCs similar to BM-MSCs. Minimal criteria have been proposed to define MSCs—and by similarity, ADSCs—by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [28]. These are: plastic adherence ability; tripotential mesodermal differentiation potency into osteoblasts, chondrocytes, and adipocytes; and immunomodulatory capability [75]. Several groups demonstrated ADSCs

multipotency showing differentiation towards various cells derived from ectoderm (epithelial cells and neurons), mesoderm (connective stroma, cartilage, fat, and bone cells), and endoderm (muscle cells, gut epithelial cells, and lung cells) [44, 77, 93, 108, 112].

Phenotypically, ADSCs characterization is still in its infancy and all attempts to discriminate clearly between them and similar cell lines have been unsuccessful. According to the literature, ADSCs' phenotypic profile is very consistent and share over 90 % of MSCs markers: CD9, CD10, CD13, CD29, CD44, CD54, CD55, CD71, CD73, CD90, CD105, CD146, CD166, and STRO-1. ADSCs also share cell surface antigens with fibroblasts and pericytes which are negative for the hematopoietic lineage markers: c-kit, HLA-DR, CD4, CD11b, CD14, CD16, CD45, CD56, CD62E, CD79, CD104, CD117, and CD106 and for the endothelial markers: CD31, CD144, and von Willebrand factor [77, 112]. The adherence to plastic and subsequent expansion of ADSCs in fetal bovine serum (FBS)-supplemented medium selects for a relatively homogeneous cell population, enriching for cells expressing a stromal immunophenotype, compared with the heterogeneity of the crude SVF [67]. The expression of CD34, MHC class I and II molecules, CD80, CD86, CD45, CD11a, CD14, CD117, HLA-DR, CDKN1B, INS, ITGA5, NOG, UTF1, WNT6, and WNT8A often decreases with culturing, whereas the expression of CD9, CD13, CD29, CD44, CD63, CD73, CD90, CD105, CD166, ACTG2, ACVR1, BMPR2, CTNNB1, CCNE1, CDH1, COL6A2, HSPA9, IL6, ITGA8, ITGB1, ITGB5, MDM2, PTEN, PUM2, SNAI2, TGFBR1, and VEGF-A tends to increase [9]. This observation results not only in a more homogeneous cell population with extended culturing [59] but also in changes in ADSCs features: CD34+ cells have a greater proliferative capacity whereas CD34-cells have a higher plastic adherence. The problem of changing surface markers during in vitro expansion cannot be satisfyingly solved at present and more detailed molecular data are necessary before a clear knowledge of the global and specific gene and protein expression profile of ADSCs (prerequisite for a highly effective cell therapy) can be achieved.

Based on β-galactosidase activity, ADSCs have been shown to exhibit telomerase activity similar to BM-MSCs that, although lower than that in cancer cell lines, indicates maintenance of the capacity for self-renewal and proliferation even after transplantation [45]. However, their capacity for in vitro expansion is limited: a recent study investigated the fundamental changes of ADSCs in long-term culture by studying the morphological feature, growth kinetic, surface marker expressions, expression level of the senescence-associated genes, cell cycle distribution, and β-galactosidase activity. The morphology of ADSCs in long-term culture showed the manifestation of senescent feature at passage (P) 15 and P20 and all the results showed that in vitro culture beyond P10 favors senescence pathways and therefore limits their clinical use [80].

3 ADSCs Isolation and Culture Procedures

The simple surgical procedure, the easy and repeatable access to the subcutaneous adipose tissue, and the uncomplicated enzyme-based isolation procedures make adipose tissue a very attractive source of stem cells for researchers and clinicians [18, 76]. However, in addition to the lack of standardization in defining what is meant by ADSCs, one of the most significant issues limiting the interpretation of results and clinical progression of ADSCs research is the lack of consensus on the way of isolating these cells. ADSCs prepared from human lipoaspirate differ in purity and molecular phenotype, with many groups using different heterogeneous cell preparations. Therefore, there is a strong need for optimization of isolation and propagation procedures for potential subsequent clinical use.

The state-of-the-art stem cell isolation technique includes several common steps to process cells from adipose tissue: washing; enzymatic digestion/mechanical disruption; and filtration and centrifugal separation for isolation of cells that can be used directly, cryopreserved, or expanded for the generation of ADSCs.

Isolated ADSCs are typically expanded in monolayer culture on standard tissue culture plastics with a basal medium containing 10 % FBS [91]. Today, efforts are being made to work under serum-free conditions and the use of serum-replacement factors, such as human platelet lysate, has become a promising technique [14]; ADSCs isolated and expanded in human serum share characteristics with cells cultivated in FBS [12]. ADSCs proliferation can be stimulated by a single growth factor such as fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), insulin growth factor-1 (IGF-1), or tumor necrosis factor-γ (TNF-γ), by platelet-derived growth factor (PDGF) via c-Jun N-terminal kinases (JNKs) activation, and by oncostatin M via activation of the microtubule-associated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) and the JAK3/STAT1 pathways [48]. ADSCs proliferation is also enhanced by multiple growth factors, which can include any of the single growth factors mentioned above supplemented by thrombin-activated platelet-rich plasma [53], human platelet lysate [46], and human thrombin [36]. Cell culture conditions markedly affect ADSCs gene expression profiles, with particular reference to the medium used and to the mechanophysiological environment (e.g., three-dimensional culture, the imposition of mechanical force on the cells and the degree of oxygenation).

Finally, it is believed that culturing ADSCs in the form of multicellular aggregates (MAs) can improve cell–cell and cell–matrix signaling. ADSCs within the MAs maintain the capacity to adhere to tissue culture plastic, as well as to proliferate and differentiate along multiple lineages. MA methods are also capable of reducing the donor-to-donor variability that exists when ADSCs are cultured in monolayer [4]. MAs provide ADSCs with a three-dimensional growth microenvironment more similar to that found in vivo and this enhances proliferation, differentiation, and angiogenesis [10].

In all protocols, however, cell yield strictly depends on individual variability. Oesayrajsingh-Varma and co-workers found that the average yield of stromal vascular cells through standard conditions was approximately $0.5–0.7 \times 10^6$ cells/g adipose tissue, with about 82 % viability after the extraction procedure, which was later confirmed by independent groups [70]. Harvesting techniques might reduce this yield, even if metabolic characteristics and cell viability seem not to differ when comparing standard liposuction with syringe aspiration of adipose tissue [88]. Other factors affecting yield variability are whether the adipose tissue is in the form of a solid resection or a liposuction sample and whether tumescent solution is used for liposuction [70].

Factors such as donor age, donor body mass index (BMI; [101]), type (white or brown adipose tissue) and localization (subcutaneous or visceral) of the adipose tissue, type of surgical procedure, culturing conditions, exposure to plastic, plating density, and media formulations might influence both proliferation rate and differentiation capacity of ADSCs. The younger the donor, the greater the proliferation and cell adhesion of the ADSCs, whereas cells gradually lose their proliferative capacity with subsequent passaging [41]. Clinical studies examining subcutaneous adipose tissue from 12 to 52 donors have reported reduced ADSCs adipogenesis, angiogenesis, osteogenesis, and/or proliferative capacity as a function of advancing donor age [64]. But the situation is much more complex inasmuch as several studies reported the absence of a significant correlation between cell yield and the age (and BMI) of patients, with regression lines showing null correlation [7, 69].

Concerning the source of adipose tissue, a detailed comparison of five different subcutaneous depots determined that ADSCs isolated from the arm and thigh best maintained adipogenic potential as a function of advancing age [83]. Moreover, ADSCs harvested from superficial abdominal regions are significantly more resistant to apoptosis than those harvested from the upper arm, medial thigh, trochanteric and superficial deep abdominal depots [83]. Further studies in larger cohorts will be necessary before patient demographics can be used to predict the functionality and recovery of SVF cells and ADSCs from donors as well as the relative utility of specific depot sites.

Other reports investigated differences due to different isolation procedures: different collagenase batches and centrifugation speeds can cause the isolation of different cell subsets [106]. Several enzymes were compared with respect to yield of nucleated cells and precursor cells. However, again, results showed that inter-donor variability is greater than differences between individual enzymes [34, 106]. The enzymes used to disrupt lipoaspirate tissue might contain, in their crude form, contaminating amounts of endotoxin, other peptidases, and xenoproteins [103]. To overcome this problem, functional ADSCs can be isolated directly from lipoaspirate fluids by mechanical devices without the need for collagenase digestion [40]. This reduces adipose cell cluster size, while eliminating oil and blood residues from the final cell product suspension. The reduced particle size of adipose tissue allows stem cells to creep out of the tissue and within 7 days they are ready for the first P. The development of an efficient and reproducible mechanical-based tissue disruption

process would remove the need for enzyme reagents and this merits further investigation.

4 Differentiation Potential of ADSCs

The broad range of clinical applications for ADSCs largely depends on their potential for differentiation and on their ability to migrate and to recruit endogenous stem cells from the niches. There are numerous scientific publications demonstrating that ADSCs possess the potential to differentiate towards a variety of cell lineages both in vitro and in vivo.

Numerous pre-clinical studies, defining various potential applications for ADSCs in human therapies, have indeed documented the ability of ADSCs to repair not only mesodermal tissues, but also ectodermal and endodermal tissues or organs, in the fields of gastroenterology, neurology, orthopedics, reconstructive surgery, and related clinical disciplines [28, 111]. The first clinical trials with SVF cells and ADSCs are ongoing, in the form of phase I (e.g., myocardial infarction, skin ulcer, or graft versus host disease), phase II (e.g., in rectovaginal fistula), phase III (e.g., enterocutaneous fistula), and phase IV (e.g., breast reconstruction) studies.

ADSCs are of mesodermal origin but their differentiation process can be switched to ectoderm and endoderm lineage cells by overexpression of lineage-specific transcription factors [111]. The processes of proliferation, allocation, and lineage-specific terminal differentiation are regulated by a complex interplay involving stem cell transcription factors (molecular rheostats), cell-specific transcription factors, and a wide variety of cellular kinases, growth factors, and receptors.

ADSCs capability to differentiate towards adipogenic lineages has implications for breast soft tissue reconstruction after tumor surgery, for breast cancer, breast asymmetry, and soft tissue and subdermal defects, after trauma, surgery, or burn injury. Confirmation of differentiation into adipocytes has usually been performed by staining with oil red O or Nile red and by analysis of the expression of adipocyte-specific genes. ADSCs-derived adipocytes develop important features known from mature adipocytes, such as lipolytic capacity upon catecholamine stimulation, anti-lipolytic activity mediated by adrenoceptors, and the secretion of typical adipokines, such as adiponectin and leptin [29]. Although several transcriptional key events regulating the differentiation of preadipocytes into mature adipocytes have been identified in the last decade (PPARγ), master genes committing the multipotent mesenchymal stem cell to adipoblasts are still awaiting discovery. As the differentiation of ADSCs into adipocytes is not in any doubt and as there is a strong demand in reconstructive and cosmetic surgery, progression of related clinical treatments and trials is further advanced than for other differentiation lineages.

ADSCs can be used for skeletal regeneration of inherited and tumor- or trauma-induced bone defects, thanks to their osteogenic differentiation. Human [43] and mouse [71] ADSCs can acquire typical osteoblastlike differentiation hallmarks, such as mineralized ECM production (calcium phosphate deposits), expression of the osteoblast-associated proteins osteocalcin and alkaline phosphatase (ALP), and response to mechanical loading. Following osteogenic differentiation, ADSCs can acquire even bone cell-like functional properties, such as responsiveness to fluid shear stress [52] and increase their expression of both ALP and mechanosensitive genes, such as osteopontin, collagen type I, and COX-2 after mechanical loading. Menin, Shh, and Notch-1 were reported to be involved during the acquisition of an osteogenic phenotype [8]. However, the (transcription) factors that initially commit ADSCs to the osteocytic lineage are widely unknown. Gene or protein expression profiles specific for osteoblasts are also lacking. Demonstration of ADSCs' differentiation into an osteogenic lineage is more complex than adipogenic differentiation. The simplest method reported is staining for calcified ECM components. Alizarin red is now commonly being used to evaluate the presence of calcium-rich deposits produced by cells in culture, along with naphthol fast blue or similar to stain for ALP enzyme activity. However, these methods do not specifically demonstrate differentiation of ADSCs into an osteogenic lineage.

ADSCs chondrogenic commitment is relevant for joint and disc defects repair and for plastic reconstruction of ear and nose defects. The molecular master regulators that allocate ADSCs to the chondrogenic lineage are widely unknown with a role for brachyury, bone morphogenetic protein (BMP)-4, transforming growth factor-3 (TGF-3), and Smad-1, -4, and -5. Treating ADSCs with recombinant BMP-7 stimulates chondrogenic differentiation and upregulates aggrecan gene expression [50], the predominant large chondroitin sulfate proteoglycan, a marker protein for chondrogenic differentiation. Overall, demonstration of chondrogenic differentiation of ADSCs faces the same problems as osteogenic differentiation. The most basic method used to demonstrate ADSCs differentiation into a chondrogenic lineage is staining for increased expression of proteoglycans using Alcian Blue or Safranin-O. Positive staining does not demonstrate differentiation of ADSC into cells capable of forming cartilage tissue; it simply shows the cells' increasing expression of proteoglycans. Confirmation of chondrogenic differentiation would ideally include mRNA analysis of differentiated cells for expression of cartilage-specific transcripts and analysis of the ECM produced by differentiated cells for cartilage-specific proteins.

Given their myogenic and cardiomyogenic properties, ADSCs are useful for muscle reconstruction after trauma and surgery, dystrophic muscle disorders, heart muscle regeneration, and functional improvement after myocardial infarction or heart failure. Cultured ADSCs have the potential for differentiation into a cardiomyocytelike phenotype with specific cardiac marker gene expression and pacemaker activity [74]. However, the myogenic potential of ADSCs may be harvested in the treatment of Duchenne muscular dystrophy, an inherited genetic disorder characterized by progressive degeneration of skeletal muscle. In vivo murine studies have shown that the implantation of ADSCs into dystrophin-deficient,

immunocompetent mice resulted in restoration of dystrophin expression, both in the muscle at the site of injection and in adjacent muscles over the long term [25].

ADSCs have been shown to be useful for neovascularization and, therefore, for ischemic diseases. As far as the differentiation into cells of the mesodermal lineages and regeneration of mesodermal tissues is concerned, ADSCs can differentiate as well as into tenogenic and periodontogenic lineages [39].

Given their ability to differentiate both morphologically and functionally into neurons, they are currently under investigation for neurological diseases, for brain injury, stroke, neuronal protection, and peripheral nerve injury. Cell population obtained by the cultivation in neurodifferentiative medium is often a mix of cells expressing one or more neurospecific markers. The most cited proteins include: myelin basic protein, nestin, beta-3 tubulin, S100, and glial fibrillar acidic protein (GFAP; [108]).

Finally, it has been shown that ADSCs can differentiate into endoderm lineage cells. Several reports have shown that ADSCs have the potential to differentiate into hepatocytes as indicated by the presence of hepatocyte growth factor (HGF) and FGF-1 and -4 [3]. ADSCs can be induced to become not only hepatic cells, critical for chronic liver failure, hepatic regeneration, or even hepatocyte transplantation, but also pancreatic/endocrine insulin-secreting cells, relevant for type 1 diabetes mellitus. Timper and co-workers [96] were successful in differentiating human ADSCs into cells with a pancreatic endocrine phenotype using the differentiation factors activin-A, exendin-4, HGF, and pentagastrin. The differentiated cells expressed the endocrine pancreatic hormones insulin, glucagon, and somatostatin.

Methods used to differentiate ADSC down these different lineages are still relatively generic and unsophisticated in many publications. The lack of control cells such as differentiated mesenchymal cells can make it difficult to determine whether the "differentiation" observed is unique to ADSCs or a property shared with other MSCs. The field would clearly benefit from more standardized disclosure of the experiments conducted. Such disclosure might include better characterization of the starting cell populations, especially the degree of purity of the ADSCs and their cell surface molecular profile by flow cytometry; more rigorous sets of molecular markers to confirm lineage commitment and integration of standardized methods for their detection at the protein or mRNA level, based on positive control tissues such as bone; wider use of control mesenchymal cells or tissue such as differentiated human fibroblasts to confirm unique properties of ADSCs; and functional assays that link through to clinical applications, such as the ability to accumulate lipid for adipocytes or to generate 3D connective tissue for other lineages. Only with reliable, standardized basic science research can any real clinical progression be achieved [62].

5 Neural Differentiation of ADSCs

A successful protocol for obtaining cells with a well-defined neural phenotype from adipose tissue is described, and it includes three major steps [102]:
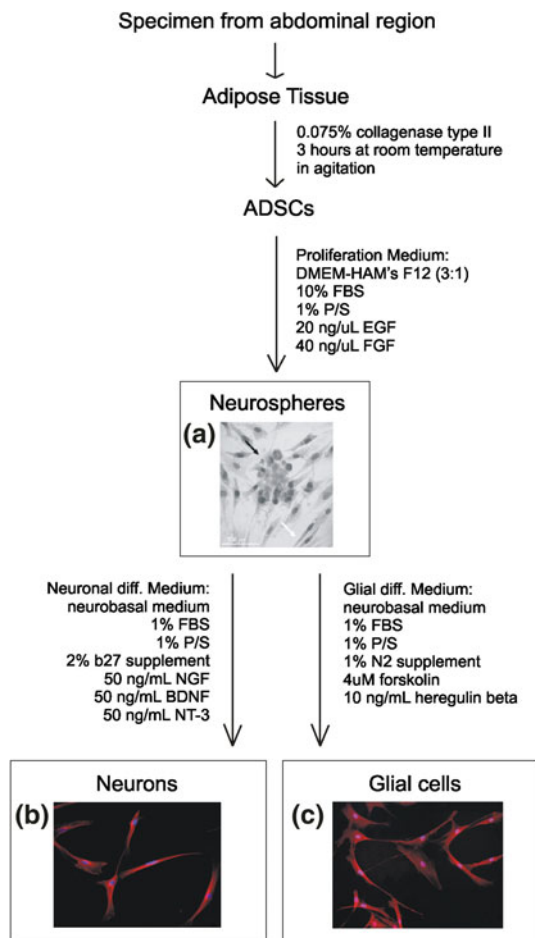 (a) Isolation of ADSCs
  (b) Neurospheres generation
  (c) In vitro neural differentiation and characterization

5.1 Isolation of ADSCs

The first step for the isolation of ADSCs involves the processing of adipose tissue. Adipose tissue samples are typically obtained from the abdominal region of patients undergoing abdominoplastic surgery, after their fully informed consent. Cellular extraction is performed according to previously published protocols for adipose tissue [1]. For the isolation of ADSCs, the subcutaneous adipose tissue is minced with forceps, and digested with 0.075 % collagenase type II in Hank's balanced salts solution (HBSS) for three hours at room temperature with gentle agitation. At this point, the enzymatic activity is neutralized with an equal volume of complete culture medium [Dulbecco's modified Eagle's medium (DMEM), containing 10 % FBS, and 1 % penicillin/streptomycin (P/S)]. The digestion products are then filtered through a 70 μm cell strainer and centrifuged at 1,000 rpm for 5 min.

5.2 Neurospheres Generation

The cellular pellet obtained from the digestion of adipose tissue is resuspended with proliferation medium [DMEM-HAM's F12 (3:1), 10 % FBS, 1 % P/S, 20 ng/μL EGF, and 40 ng/μL FGF] and seeded at a density of 10^5 cells/cm^2. Cells are cultured at 37 °C in humidified atmosphere with 5 % CO_2. After 7 days under these culture conditions, most of the cells adhere to the tissue culture plastic, assuming a fibroblast like phenotype that is flattened and spindle shaped. Nevertheless, a small population of expanded cells is able to organize into spheres growing in suspension. These proliferating spheres, called "neurospheres," are cell aggregates arranged in three-dimensional structures. It has been shown that the highest number of neurospheres from adipose tissue is generated in vitro when the culture medium is supplemented with serum and enriched with a combination of EGF and FGF. In addition, it is also necessary for the production of neurospheres that cells are cultured without any attachment factor [102, 109]). Once formed, floating neurospheres are passaged by harvesting and centrifugating the medium. Then, neurospheres are resuspended in proliferation medium and mechanically dissociated by vigorous trituration with a

Specimen from abdominal region

↓

Adipose Tissue

> 0.075% collagenase type II
> 3 hours at room temperature
> in agitation

↓

ADSCs

> Proliferation Medium:
> DMEM-HAM's F12 (3:1)
> 10% FBS
> 1% P/S
> 20 ng/uL EGF
> 40 ng/uL FGF

Neurospheres

(a)

Neuronal diff. Medium:
neurobasal medium
1% FBS
1% P/S
2% b27 supplement
50 ng/mL NGF
50 ng/mL BDNF
50 ng/mL NT-3

Glial diff. Medium:
neurobasal medium
1% FBS
1% P/S
1% N2 supplement
4uM forskolin
10 ng/mL heregulin beta

Neurons **(b)**

Glial cells **(c)**

Fig. 1 Schematic representation of the steps leading to neurosphere generation from ADSCs, and their subsequent in vitro differentiation towards a neural phenotype. **a** Neursopheres growing in suspension (*black arrow*) onto a carpet of fibroblastoid cells (*white arrow*). **b** βIII tubulin staining showing neuronal differentiation of ADSCs-derived neurospheres. **c** GFAP staining showing glial cell differentiation of ADSCs-derived neurospheres

Pasteur pipette. At this point, cells are cultured at high density in new culture flasks with fresh proliferation medium for an additional 7 days.

5.3 In Vitro Neural Differentiation and Characterization

In order to induce neuronal or glial differentiation, cells derived from neurospheres are seeded onto poly-D-lysine culture dishes and cultured with neuronal

differentiation medium [neurobasal medium, 1 % FBS, 1 % P/S, 2 % b27 serum-free supplement, 50 ng/mL nerve growth factor (NGF), 50 ng/mL brain-derived neurotrophic factor (BDNF), 50 ng/mL neurotrophin-3 (NT-3)] [32] or glial differentiation medium (neurobasal medium, 1 % FBS, 1 % P/S, 1 % N2 supplement, 4 μM forskolin, and 10 ng/mL heregulin β; [99]. After 14 days under these culture conditions, cells are characterized for the expression of neurospecific markers in vitro. Immunohistological analyses show that these cells are able to differentiate into cells with a neural phenotype, often showing the expression of more than one neural marker. A common protein used for the identification of neural cells is vimentin. Vimentin is the most ubiquitous intermediate filament (IF) protein and the first to be expressed during cell differentiation. Vimentin is present in a wide variety of mesenchymal cell types and in many cells from the neural crest, where it appears to maintain structural integrity and cell shape. It has recently been found that vimentin co-localize with nestin in the microglia [92]. Nestin is another protein whose expression is related to a neural phenotype. Nestin, a type VI IF protein is expressed in dividing cells during the early stages of development in the central nervous system (CNS) and peripheral nervous systems (PNS). Upon differentiation, nestin becomes downregulated and it is replaced by other tissue-specific IF proteins, that is, GFAP in glia and neurofilaments/α-internexin in neurons [66]. GFAP is a type III IF protein specifically found in mature glial cells (astrocytes; [79]; instead, neurofilaments, which are type IV IF proteins, become the principal constituents of the neuronal IF network after synaptic connections are established [105]. βIII tubulin is another well-known neuronal marker [17]. βIII tubulin is a cytoskeletal dimer abundant in the CNS and PNS where it seems to be important for maintaining neurite elongation [33]. CNPase (3′-cyclic nucleotide 3′-phosphodiesterase) is an enzyme highly expressed in oligodendrocytes of CNS and Schwann cells of PNS [54]. CNPase plays a role in tubulin polymerization and oligodendrocyte process outgrowth [57].

When treated with neuronal or glial differentiation media, ADSCs show the expression of vimentin and nestin. Neurofilaments and βIII tubulin are selectively expressed by neuronlike cells whereas GFAP and CNPase are specific markers for glial cells (Fig. 1).

6 Therapeutic Applications of MSCs

Human neurological disorders are largely caused by a loss of neurons and glial cells in the brain or spinal cord. Endogenous neural progenitors have a limited proliferative capacity and the newly generated neurons will also be prone to the same degeneration over time. For these reasons, repair of the disease process with endogenous neurons does not correct the underlying cause of the initial degeneration. Cell-based therapies are emerging as innovative approaches for the treatment of such defects. In particular, MSCs derived from a variety of tissues have been therapeutically evaluated in animal models of stroke, spinal cord injury, and multiple

Table 1 Examples of in vivo applications of MSCs to treat SCI

References	Stem cells	Differentiation	Application	Result
[24]	Human BM-MSCs	No	Human BM-MSCs were administered to rats at 3 or 7 days after SCI as a single injection or as three daily injections, either at 3, 4, and 5 days or at 7, 8, and 9 days following SCI	Better results were obtained in rats treated with three consequent daily injections of BM-MSCs at 7, 8, and 9 days, showing significantly higher motor function recovery at 14–28 days post injury. Transplanted BM-MSCs were found to migrate and incorporate into the central lesion. Moreover, human BM-MSCs were able to differentiate into oligodendrocytes, but not into cells expressing neuronal markers, indicating that human MSCs may facilitate recovery from SCI by remyelinating spared white matter tracts
[22]	Rat ADSCs	Schwann cells (SCs)	Induced SCs were transplantated in the center of rat SCI lesions 9 days after injury	Induced SCs formed a PNS-type myelin sheath on CNS axons, and showed expression of neurotrophic factors that stimulated neurite outgrowth
[5]	Rat ADSCs	Pre-differentiation into spheres and differentiation into glial cells and SCs	Spheres derived from ADSCs were transplanted into rats 1 week after spinal cord compression lesions	Pre-differentiated ADSCs well interacted with the host tissue by wrapping host axons and oligodendrocytes

Table 2 Examples of in vivo applications of MSCs to treat brain stroke

References	Stem cells	Differentiation	Application	Result
[58]	Rat BM-MSCs	No	Rat BM-MSCs were transplanted into mice striatum at 4 days after embolic middle cerebral artery occlusion (MCAO)	Intra-striatal transplanted BM-MSCs survived in the ischemic brain and improved functional recovery of adult mice even though infarct volumes did not change significantly
[19]	Rat BM-MSCs	No	Rat BM-MSCs were intravenously injected into rats 1 or 7 days after stroke, either at low-dose (1×10^6 cells) or high-dose (3×10^6 cells)	Direct intravenous delivery of high-dose BM-MSCs after 1 or 7 days from stroke led to migration of cells to ischemic infarcts, survival, and differentiation into neuronal and glial cell types, and functional improvement
[47]	Human ADSCs	Neural cells	Differentiated human ADSCs were injected into the lateral ventricle of rat brain 1 day after MCAO	Ischemic brain injury by MCAO notably increased migration of transplanted cells to the injured cortex. At 7 days after stroke, transplanted rats showed a good recovery in motor and somatosensory behavior
[47]	BDNF-transduced human ADSCs		BDNF-transduced ADSCs were intra-cerebrally grafted into MCAO rats	The extent of recovery was further improved after transplantation of BDNF-transduced ADSCs in ischemic rats. This result demonstrates that transplanted ADSCs expressing BDNF can function as useful vehicles for gene therapy in neurodegenerative diseases
[51]	Human ADSCs	No	Human ADSCs (3×10^6 cells) were administered via a tail vein into rats 1 day after intra-cerebral hemorrhage (ICH) injury	ADSCs transplantation reduced apoptosis and cerebral inflammation, and attenuated brain atrophy and glial proliferation at 6 weeks after IHC. In addition, it was found that transplanted ADSCs survived for 42 days in the IHC area, showing expression of endothelial markers, but not neural markers

(continued)

Table 2 (continued)

References	Stem cells	Differentiation	Application	Result
[72]	Human BM-MSCs	No	Human BM-MSCs (1×10^6 cells) were intravenously injected into rats 6 h after permanent MCAO	Intravenous infusion of human BM-MSCs led to a reduction in infarction volume, induction of angiogenesis, and improvement in behavioral performance
[72]	Angiopoietin-1-transduced human BM-MSCs	No	Angiopoietin-1-genetically modified human BM-MSCs (1×10^6 cells) were injected into rats 6 h after permanent MCAO	Angiogenesis and functional recovery were moderately greater after angiopoietin-1 modified BM-MSCs

Table 3 Examples of in vivo applications of MSCs to treat TBI

Reference	Stem cells	Differentiation	Application	Result
[63]	Human UCB-MSCs	No	UCB-MSCs were injected into the tail vein of rats 1 day after TBI	The injection of UCB-MSCs led the cells into the parenchyma of the injured brain, where they reduced motor and neurological deficits
[65]	Human BM-MSCs	No	Human BM-MSCs (1×10^6 cells or 2×10^6 cells) were injected into the tail veins of rats 24 h after TBI	The transplanted cells migrated into injured brain, preferentially localizing around the injury site. An improvement in functional outcome was seen, in particular with the high dose of the injected BM-MSCs. Some of these cells expressed phenotypic features of neurons and astrocytes. Nevertheless, the functional benefit obtained after human BM-MSCs transplantation was probably due to the production of the neurotrophic growth factors NGF and BDNF
[56]	Human ADSCs	Neuronal cells	ADSCs with neuronal phenotype were transplanted into rats subjected to toxic effect of 3-nitropropionic acid	Under the effect of ADSCs, the number of neurons increased twofold, the cells acquired the typical round shape, and cell edema decreased

sclerosis. These applications are possible owing to the characteristics of MSCs such as plasticity, immunoregulatory actions, immunosuppressive properties, and homing to areas of insults where they can release a wide range of tropic signals that influence surrounding tissues [35]. Indeed, critical to the success of cell-based therapies is the selection and mode of delivery of therapeutic cells.

Neurological disorders include spinal cord injury (SCI), brain stroke, traumatic brain injury (TBI), Parkinson's disease (PD), Huntington's disease, Alzheimer's disease, and multiple sclerosis. Here the in vivo applications of MSCs, with particular respect to ADCSs, in several neurological disorders are reported as described in the literature.

6.1 Spinal Cord Injury

SCI is an acute traumatic lesion of neural elements in the spinal canal, resulting in temporary or permanent sensory and/or motor deficit. Many cases of SCI are the result of motor vehicle crashes, falls, acts of violence, and recreational sporting activities [98], with the average injury age of 33 years [84]. The pathophysiology of SCI is biphasic, comprising a primary and secondary injury phase [94]. The primary injury phase refers to the injury itself, and consists of mechanical disruption of the spinal cord. The most common injury mechanism is the spinal contusion and compression due to crushing of the vertebrae. Hyperbending, hyperstretching, rotation, and laceration can also occur, however, complete transactions of the cord are rare. Although tissue damage can occur during the immediate phase, there is the rare case of permanent pathological changes. The secondary injury phase depends on the kind of damage in the early phase and on the time post injury. It includes three distinct phases: acute, intermediate, and chronic. In the acute and intermediate phases, inflammation, reactive oxygen species (ROS) production, and lipid peroxidation cause apoptosis and necrosis of both neurons and oligodendrocytes, leading to neurological deficits. The late intermediate and chronic phases are characterized by progressive degeneration, accompanied by attempts at endogenous repair [78]. Morphological changes associated with the chronic phase represent an obstacle to any cell replacement therapy, therefore cell administration occurs during the intermediate phase, that is, 7–14 days after injury [30] (Table 1).

6.2 Brain Stroke

Brain stroke refers to a series of conditions caused by the occlusion or hemorrhage of blood vessels supplying the brain. Most often, blood flow is compromised within an occluded blood vessel; less commonly, stroke results from the absence of blood flow to the entire brain due to cardiac arrest [61]. Unlike other neurological disorders, brain stroke affects different neuronal cells: apart from neurons,

oligodendrocytes, astrocytes, and endothelial cells are also damaged [82]. For these reasons, brain stroke can lead to motor, sensory, or cognitive impairments (Lindvall and Kokaia 2006). Stem cell transplantation has emerged as an experimental approach to promote recovery and restore brain function after a stroke. It is hypothesized that the mechanisms of recovery are likely due to the release of trophic factors by these cells [21], possibly promoting endogenous repair mechanisms, reducing cell death, and stimulating neurogenesis and angiogenesis, rather than neuronal differentiation [20]. Most of the pre-clinical studies on stroke transplantation use ASCs derived from the bone marrow, umbilical cord blood (UCB), or adipose tissue. Even though the long-term effects of these cells in the brain are unknown, first results support the hypothesis that transplanted stem cells after stroke provide trophic support (Table 2).

6.3 Traumatic Brain Injury

TBI is a leading cause of death and disability in the United States, especially in children and the elderly [55]. Primary brain injury results from the direct application of an external force to the cranium and intracranial contents. When these forces strain the cerebral tissue beyond its structural tolerance, injury results. The type of strain may be compressive, tensile, or shear in nature. Secondary brain damage results from the post-traumatic pathophysiological cascade that follows the initial injury and contributes to delayed tissue injury and neuronal loss. The initial mechanical disruption of the brain and its vasculature is followed during the first week by cellular damage, the development of edema, and the liquefaction of the hematoma if one is present. Nerve repair after TBI remains a challenging clinical problem; the use of ASCs could represent a new way of treatment to minimize the death of neurons and loss of neurological function after TBI (Table 3).

7 Regulatory Framework

It sounds simple to obtain sufficient numbers of cells derived from fetal or adult human tissues: isolate and/or expand the stem cells, and then transplant an appropriate number of these cells into the patient at the correct location. However, translating basic research into routine therapies is a complex multistep process that necessitates product regulation [13].

To demonstrate safety and efficacy, the translational development of cell-based therapy requires rigorous large-animal experimentation but inherent limitations with rodent experimentation are largely recognized. In addition to causing a potential lack of efficacy that could be encountered with chemical drugs (e.g., absence of the target, differences in metabolism, pharmacokinetics), human cell-based products elicit an immune reactive response in the host that rejects these

cells. Thus, the cells must be tested in immunodeficient animals. Nude or severe combined immunodeficiency (SCID) mice have been extensively used for this purpose, but they bear residual immune cells that could interfere with the human cells and thus bias the results. Thus, NOG mice or other very immunocompromised mice should be used. However, immunocompromised mice represent an aberrant immune context for many applications of cell-based therapy. Furthermore, mice may not be a relevant pathological model with their reduced life span as compared with humans and their size, which leads to difficult functional evaluation. Despite these limitations, regulatory agencies require such validations and a wealth of data have been published evaluating the safety and efficacy of ADSCs.

Nevertheless, there are still open questions concerning the best ways to maintain quality when these cells are administered as medicinal products.

The European directives (Directive 2001/83/EC; 2003/63/EC; and Regulation 1394/2007) on human cell-based medicinal products acknowledge that, given their complexity, conventional clinical and nonclinical pharmacology and toxicology studies may not be appropriate for cell-based medicinal products. For the same reason, regulatory authorities offer investigators their advice to fine-tune the process from the early beginning on.

In Europe, ADSCs are classified as advanced therapy medicinal products (ATMPs) [87]. ATMPs include gene therapy medicinal products, somatic cell therapy products (as defined in Directive 2001/83/EC), and tissue-engineered products. Cells fall under this regulation in case they have been subjected to substantial manipulation, resulting in a change of their biological characteristics, physiological functions, or structural properties. Moreover, ATMPs refers to cells or tissues that are not intended to be used for the same essential functions in the recipient as in the donor.

Cell-based medicinal products can be considered as a single production, more similar to a graft than to a chemical drug and often involve a limited amount of cell samples, mostly to be used in a patient-specific manner. The highly repetitive and controlled methods employed in the manufacture of cellular products are a critical factor because these products are partly defined by reference to their method of manufacture. Therefore, all the personnel, as well as the physical structures and the materials, involved in each step must be in accordance with good manufacturing practice (GMP) and within an accepted quality system.

For this reason, clinical centers aiming to apply ADSCs-based therapies require access to a GMP facility, supported by a highly specialized staff of technicians and qualified persons. This greatly limits the potential applications of ADSCs-based therapies to larger clinical centers capable of housing such facilities and thus results in a cost-ineffective therapeutic approach.

Different regulatory bodies around the world provide GMP guidance (EudraLex—Volume 4 GMP Guidelines). The aim of all laws on cellular medicinal products is the guarantee of their overall safety, with a positive balance between risks and benefits for patients. The risk posed by the administration of a cell-based medicinal product, in fact, is highly dependent on the origin of the cells, the

manufacturing process, the noncellular components, and on the specific therapeutic use. The following general risk criteria can be used in the estimation of the overall risk of the product: origin (autologous-allogeneic), ability to proliferate and differentiate, ability to initiate an immune response (as target or effector), level of cell manipulation, mode of administration (ex vivo perfusion, local, systemic), duration of exposure (short to permanent), and availability of clinical data on or experience with similar products (EMEA/CHMP/410869/2006).

As stated above, to reduce these risks, cell-based medicinal products intended for clinical use must be produced via a robust manufacturing process governed by a quality control sufficient to ensure a consistent and reproducible final product. Structure layout and design must minimize the risk of errors and permit effective cleaning and maintenance in order to avoid cross-contamination, buildup of dust or dirt and, in general, any adverse effect on the quality of products. The manufacture must be carried out in clean areas (class A in B, in C, in D), entry to which is through airlocks for personnel and/or for equipment and materials. In order to meet "in operation" conditions, these areas must be designed to reach certain specified air-cleanliness levels in the "at rest" occupancy state. Clean rooms and clean air devices are routinely monitored in operation and the monitoring locations are based on a formal risk analysis study. Standard operating procedures (SOP) must be written and concern manufacturing and release of cell-based products, starting materials, traceability, and quality controls. Viral and transmissible spongiform encephalopathy (TSE) safety of the cells and raw materials has to be addressed to minimize the risk of contamination. When bovine serum is used, the recommendations of the Note for Guidance on the "Use of Bovine Serum in the Manufacture of Human Biological Medicinal Product" must be followed.

Moreover, because cell viability has to be preserved in the final product, cell-based medicinal products cannot be sterilized at the end of the process: they have to be produced aseptically and their sterility must be proven. By using human and/or xenogenic material, there is the potential for adventitious agent contamination and therefore all cell products for human clinical applications must be proven to be free of bacterial, endotoxin, mycoplasma, and viral (B19, cytomegalovirus, Epstein–Barr virus, hepatitis B and C, human immunodeficiency viruses 1 and 2, as well as human T cell leukemia viruses 1 and 2, EMEA/CHMP/410869/2006) contamination. For the European Union and United States, criteria for these test procedures differ.

The adipose tissue donors may themselves be carriers of infectious agents and the possibility of viral infections has to be eliminated prior to sample acceptance. In general, the starting material is a critical issue and is evaluated through common donor eligibility criteria, such as age and viral testing.

Once produced, GMP grade products must be maintained in liquid nitrogen vapor phase storage containers that in turn remove any risk of cross-contamination between individual containers. A variety of studies investigated the effects of storage conditions and cryopreservation methods and media [97], demonstrating that MSCs can be cryopreserved and thawed without loss of function [15]. Cryopreservation gives the only opportunity to perform time-consuming release

tests prior to clinical application of the cells, hardly possible to achieve when the cell product is intended for immediate release.

Given the complexity of GMP requirements, regulatory compliance is challenging not only for cell-therapy laboratories but also for the entire scientific community. Regulatory agencies should establish, standardize, and harmonize translational protocols to guarantee safety of cell-based medicinal products and, on the other hand, should assure that all available treatments can reach the patients.

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Migratory Properties of Mesenchymal Stem Cells

Thomas Dittmar and Frank Entschladen

Abstract Mesenchymal stem cells raise great expectations in regenerative medicine due to their capacity to regenerate damaged tissues, thereby restoring organ tissue integrity and functionality. Even though it is not yet clear how mesenchymal stem cells are guided to injured tissue it is generally assumed that the directed migration of these cells is facilitated by the same soluble factors that also recruit immune competent cells to inflamed tissue areas. Tumor tissue represents another type of (chronically) inflamed tissue and because of that mesenchymal stem cells are highly attracted. Although some data indicate that esenchymal stem cells might have a beneficial effect on tumor growth due to anti-tumor effects the plethora of data suggest that tumor tissue recruited mesenchymal stem cells rather promote tumor growth and metastasis formation. Nonetheless, the enhanced tumor tropism of mesenchymal stem cells makes them ideal candidates for novel anti-cancer strategies. Like Trojan Horses genetically modified mesenchymal stem cells will deliver their deadly cargo, such as anti-tumor cytokines or oncolytic viruses, into cancerous tissues, thereby destroying the tumor form within. In this chapter we will summarize the current concepts of genetic modification of mesenchymal stem cells for future anti-cancer therapies.

Keywords Mesenchymal stem cells · MSCs · Inflammation · Tumor tropism · Trojan horse

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1 Introduction

Only very few specialized cells within an adult human are capable of autonomous migration. Physiologically, these cells are leukocytes, fibroblasts, and stem cells, although the pathological migration of cancer cells also occurs during tumor progression. Among these groups, stem cells are probably the less investigated cells. However, in a comparative view of these migrating cells, mesenchymal stem cells (MSCs) have an interesting role because they combine certain characteristics of both leukocytes and cancer cells. On the one hand, the migration of MSCs is strongly controlled by chemokines. This group of ligands to G protein coupled receptors consists of inflammatory chemokines (e.g. interleukin-8) and constitutive chemokines (e.g. stromal cell-derived factor-1; SDF-1), which has been characterized to cause chemotactic responses in leukocytes and guide them to certain places of need and action. Meanwhile, cancer cells and stem cells have been shown to use the chemokine system for their localization within the body as well, as will be discussed with in detail later. On the other hand, the morphology of migrating tumor cells resembles that of MSCs, and the transition of carcinoma cells from an epithelial to a mesenchymal morphology (epithelial-mesenchymal transition; EMT) is under discussion to be an important event for the onset of migratory activity, which in turn is an essential prerequisite for invasion and metastasis formation. EMT and its opposite transition MET physiologically play a central role in embryonic development, when differentiating epithelial cells lose their apical-to-basal polarity (EMT), leave the tissue network, migrate to a certain place, and retransform from mesenchymal to epithelial cells (MET). In tumor progression, a similar process might hold true for the formation of metastases.

EMT is characterized by changes in the expression of several surface molecules. Most prominently, E-cadherin is down-regulated [1, 2]. E-cadherin is a homotypic intercellular adhesion molecule. It is obvious that a down-regulation of this surface receptor facilitates the emigration of cancer cells from the primary tumor mass. Furthermore, EMT is histopathologically characterized by the expression of vimentin [3, 4] and loss of the expression of cytokeratins 5/6 [4, 5]. However, is EMT really an essential prerequisite for invasion and metastasis formation? A striking argument against this hypothesis is the fact that cell lines, which are not described to be "post-EMT," do also show migratory activity and form tumors in mice. For example, we have investigated the three human breast carcinoma cell lines MCF-7, MDA-MB-231 and MDA-MB-468 for their migratory activity [6]. MCF-7 is an estrogen receptor–positive, luminal-like cell line not expressing vimentin [7, 8]. MDA-MB-231 cells are basal-like and are classified as post-EMT because they express vimentin but not the cytokeratins 5/6 [8–10].

MDA-MB-468 cells are basal-like, too, but these cells express the cytokeratins 5/6 and not vimentin [11, 12]. Although these cells are classified to different subgroups with distinct EMT status, all of these cells show similar migratory activity [6] and are described to be metastatogenic in nude mice [13–15].

However, besides these parallels between mesenchymal (stem) cells and tumor cells, MSCs are likely to have further important functions in cancer, which we discuss in the following paragraphs.

2 MSCs as Therapeutic Options in Cancer Treatment

A well-known phenomenon of mesenchymal stem cells is their marked tumor tropism, although it still remains unclear how these stem cells are recruited to cancerous tissues. One possible explanation might be attributed to the fact that inflammatory conditions are a positive trigger for MSC recruitment [16–22] and that tumor tissue resembles chronically inflamed tissue [23–25]. In this context, tumor tissue has been compared to "wounds that do not heal" [26]. Because of the tumor tropism and the fact that MSCs can be easily modified (e.g., due to lentiviral transfection), this particular stem cell population has been chosen as an adequate cellular tool for therapeutic options in cancer treatment. Herein, we summarize the current concepts on how tumors and even metastases can be treated by genetically modified MSCs. We also present the dark site of MSCs—namely how this stem cell population triggers tumor progression.

2.1 Recruitment of MSCs to Inflamed Tissues

Little is known about how MSCs are recruited to injured tissues. It is generally assumed that MSCs most likely share the properties of immune cells and thus respond to the same chemokines as immune cells [22]. In fact, MSCs express a plethora of chemokine receptors, including CCR1–CCR10, CXCR1–CXCR6, CX3R1, and XCR1 [22] as well as c-met [27]. However, research has been published indicating that ex vivo cultivation of MSCs had a marked effect on the chemokine receptor expression pattern of the cells [28]. For instance, ex vivo cultivation of MSCs, which were positive for CCR1, CCR7, CCR9, CXCR4, CXCR5, and CXCR6, for 12–16 passages was associated with both a loss of surface expression of all chemokine receptors and lack of chemotactic response to chemokines [28]. In addition to long-term cultivation, culture conditions also could have an effect on the cells' responsiveness to chemokines. Our data showed that murine Lin$^-$ c-kit$^+$ hematopoietic stem/progenitor cells (HSPCs) cultivated with a combination of Flt3-ligand, SCF, thrombopoietin (TPO), and interleukin (IL)-11 (FSTI) showed a high migratory SDF-1α response, whereas cells cultivated with SCF, TPO, and IL-11 (STI) did not react to SDF-1α stimulation with an

elevated locomotory activity, even though STI-cultured cells exhibited slightly higher CXCR4 levels than FSTI-cultured murine Lin⁻ c-kit⁺ HSPCs [29]. Findings of Sorti et al. revealed a heterogenous expression of chemokine receptors on bone marrow-derived MSCs (BM-MSCs) [30]. Thereby, a small percentage of cells were positive for CCR1 and CCR7, whereas a higher percentage of cells, but not all cells, were positive for CXCR4, CXCR6, and CXC3CR1 [30]. However, because BM-MSCs were cultured prior to analysis, it remains unclear whether BM-MSCs were per se heterogeneous in their chemokine receptor expression pattern or whether this was a cultivation-dependent effect. In addition to long-term cultivation of MSCs and the applied culture conditions, alterations in MSC surface receptor expression patterns further depend on the confluency of the cells, the site of isolation, and the incubation environment (normoxic conditions versus hypoxic conditions) (for review see: [31]).

Son et al. demonstrated that bone marrow and cord blood MSCs express both CXCR4 and c-met receptor, were strongly attracted by stromal cell-derived factor-1α (SDF-1α) and hepatocyte growth factor (HGF), and were chemoinvasive across the reconstituted basement membrane Matrigel. These findings suggest that the CXCR4/SDF-1α and c-met/HGF axes, along with matrix metalloproteinases (MMPs), may be involved in the recruitment of expanded MSCs to damaged tissues [27]. This would be in agreement with data of Houghton et al. who demonstrated that gastric cancer originated from bone marrow-derived cells [32]. Comparison of MSCs and hematopoietic stem cells (HSCs) showed that only MSCs, but not HSCs, acquired a gastric mucosal cell-gene expression [32]. Conjointly, Western Blot analysis performed on gastric mucosa from *H. felis*-infected mice (12 and 16 months after infection) revealed a substantial upregulation of SDF-1α and stem cell factor (SCF) as compared with uninfected age-matched controls [32]. Thus, it can be assumed that MSCs were recruited from bone marrow to gastric mucosa tissue via the CXCR4/SDF-1α axis. The interplay of CXCR4 and SDF-1α has also been suggested to be involved in the recruitment of MSCs in a tumor context. Thereby, MSCs do not only respond to this chemokine with an enhanced chemotaxis, but they may also adopt a so-called carcinoma-associated fibroblast (CAF) phenotype. In vitro data provided evidence that MSCs can assume a functional CAF phenotype concomitant with sustained SDF-1α expression, as well as promote tumor cell growth in vitro and in vivo when exposed to tumor-conditioned media [33]. A similar effect has been reported for the recruitment of MSCs into injured heart tissue upon myocardial ischemia/reperfusion. Recruitment of MSCs not only depended on the interplay of monocyte chemoattractant protein-1 (MCP-1/CCL2) and CCR2, but also on the intracellular adaptor molecule FROUNT, which interacts with CCR2 [34]. FROUNT activation resulted in CCR2 clustering, reorganization of the actin cytoskeleton, and MSC polarization [34]. Moreover, MCP-1/CCL2-activated MSCs expressed increased SDF-1α levels, which, in analogy to MSC-derived CAFs, might attract additional cells to the injured tissue, including immune cells and MSCs [34]. Thus, recruitment of MSCs concomitant with MSC activation/differentiation—thereby resulting in SDF-1α secreting cells—represent a kind of a self-energizing process. It

would be of interest to investigate whether such a process also takes place in a tumor context because the interplay of CCR2 and MCP-1/CCL2 plays a pivotal role in directing MSCs to primary breast cancer cells [35]. The addition of an MCP-1/CCL2 blocking antibody significantly impaired MSC migration to breast carcinoma cells, clearly showing the correlation between secretion of MCP-1/CCL2 by breast tumor cells and the CCR7-dependent chemotaxis of MSCs. Additionally, it was noted that serum MCP-1 levels were significantly higher in postmenopausal patients with breast cancer than in age-matched controls [35]. Whether this may point to more severe breast cancer disease in these afflicted patients is not clear. Nonetheless, these data show that MSCs are effectively attracted by breast cancer tissue, which in turn is useful knowledge for MSC-based anticancer therapies. An increased migration of MSCs was further observed in irradiated 4T1 murine mammary tumor cells in comparison to unirradiated 4T1 cells [36]. Irradiated 4T1 cells exhibited increased expression levels of transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), and platelet-derived growth factor-BB (PDGF-BB). Unfortunately, the authors did not perform appropriate experiments to study whether MSCs were attracted by these secreted factors. On the other hand, studies on gliomas revealed that these tumors recruit MSCs by secreting a multitude of angiogenic cytokines, including VEGF and TGF-β1 as well as interleukin-8 (IL-8) and neurotrophin-3 (NT-3) [37]. In any case, the chemokine receptor CCR2 was markedly upregulated in MSCs exposed to irradiated tumor cells [36]. Conjointly, MCP-1/CCL2 was highly expressed in the parenchyma of murine 4T1 tumors in vivo, suggesting that MSCs were recruited to irradiated tumor cells via MCP-1/CCL2/CCR2 signaling [36]. This is further supported by data demonstrating that inhibition of CCR2 led to a markedly decreased MSC migration in vitro [36]. Similar data were provided by Zielske et al. in a recent report [38]. Here, MSCs were recruited to irradiated tumors in a dose-dependent manner [38]. Likewise, MCP-1/CCL2 levels were modestly elevated in irradiated tumors [38]. Even though these data nicely illustrate the correlation between tissue damage and recruitment of MSCs and further point to the possible increased therapeutic efficacy of genetically modified MSCs due to the increased tumor tropism, it has to be taken into account that such strategies might also have risks for the afflicted patients. Because MSCs play a crucial role in wound healing and tissue regeneration processes, as well as possess immune-suppressive properties, the enhanced tumor tropism of MSCs towards irradiated tumors might have a diametric effect on the efficacy of cancer therapy. We will discuss this point in the next part of this chapter.

Further soluble factors that directly mediate MSC chemotaxis and recruitment to damaged tissue include CCL21 [16], IL-8 [37, 39, 40], and CXC3L1 [30]. CCL21 is secreted by injured keratinocytes [16]. Intradermal injection of CCL21 increased the migration of MSCs concomitant with a greater acceleration of wound repair in an animal model [16]. Ringe et al. observed a dose-dependent chemotactic activity of BM-MSCs towards SDF-1α and IL-8 [39]. Interestingly, the authors also observed that BM-MSCs expressed CCR2, but the cells did not respond to MCP-1/CCL2 stimulation with an increased migratory activity [39].

These findings are opposed to the data summarized previously showing that the chemotaxis of MSCs was positively triggered by MCP-1/CCL2 [34, 35]. Whether these differences might be attributed to a cell culture-dependent effect or another mechanism remains unclear. An IL-8 dependent recruitment of MSCs was further observed in a glioma context. Secretion of a multitude of angiogenic cytokines, including IL-8, by glioma cells actively attracted MSCs to cancerous tissue [39]. Irradiation of glioma cells resulted in increased IL-8 expression levels, which were further associated with an upregulation of CXCR1/CXCR2 on MSCs, thereby enhancing the tumor tropism of MSCs [40].

In addition to chemokines, several studies demonstrated that growth factors can trigger the migration of MSCs, including PDGF-BB, VEGF, and TGF-β1 [36, 37]. Beside these growth factors, insulin-growth factor-1 (IGF-1), epidermal growth factor (EGF), and PDGF-AB also enhance the migration of MSCs [41, 42]. Interestingly, IGF-1 does not only induce migration of MSCs per se [42], but also triggers the migratory activity of MSCs in response to SDF-1α due to up-regulation of CXCR4 [41]. In accordance to previously mentioned data summarizing that the migratory properties of MSCs can depend on culture conditions, Ponte et al. showed that BM-MSCs exhibited an altered migratory behavior to chemokines and growth factors upon overnight incubation with tumor necrosis factor-α (TNF-α) [42]. Thereby, TNF-α increased CCR2, CCR3, and CCR4 expression, which was correlated with enhanced RANTES membrane binding as well as increased RANTES-mediated migratory activity of the cells [42]. Interestingly, TNF-α stimulated BM-MSCs also showed an increased susceptibility towards SDF-1α despite unaltered SDF-1α surface binding and CXCR4 expression [42]. Because TNF-α ultimately activates NF-κB, thus inducing gene expression, it can be speculated that the altered migratory behavior of TNF-α cultured BM-MSCs towards SDF-1α were attributed to alterations in the gene expression pattern of the cells, which might have an impact on the kinetics on the CXCR4/SDF-1α induced signal transductions cascades.

2.2 MSC and Tumor Progression

Several studies have provided evidence that MSCs are actively recruited to various tumors, including breast carcinoma [38, 43, 44], colon carcinoma [38, 45], and gliomas [46–48], as well as to lung metastases [49, 50]. Even though some data indicate that MSCs might have a beneficial effect on tumor growth due to anti-tumor effects, more and more data suggest that recruitment of MSCs to tumor tissues may be involved in cancer progression because of mediating tumor growth and metastasis formation.

Qiao et al. demonstrated that the latent time for tumor formation was prolonged and that the tumor size was smaller when SCID mice were injected with H7402 hepatoma cells and an equal number of Z3 human MSCs [51]. Co-cultivation of both cell populations revealed a decreased proliferation but increased rate of apoptosis, as well as a downregulated expression of Bcl-2, c-Myc, proliferating cell nuclear

antigen (PCNA), and survivin in H7402 cells [51]. A similar effect of MSCs was observed for MCF-7 breast cancer cells, whereby the MSC-mediated inhibition of MCF-7 proliferation was associated with decreased NF-κB and p-IκBα levels in the breast cancer cell line [52]. Likewise, β-catenin levels were decreased in MCF-7 breast cancer cells being cultured in MSC-conditioned media, which was most likely attributed to increased levels and secretion of dickkopf-1 (Dkk-1) by MSCs [53]. In fact, neutralization of Dkk-1 (either by a blocking antibody or by siRNA) attenuated the inhibitory effect of MSCs on MCF-7 cells, whereas Dkk-1 overexpression enhanced the inhibitory effect [53]. Because decreased β-catenin levels were also observed in H7402 hepatoma cells co-cultured with MSCs or being cultured in MSC-conditioned media [51], it can be assumed that MSCs impair tumor proliferation by inhibition of the Wnt pathway. Another mechanism by which MSCs may impair tumor cell proliferation might be the MSC-mediated upregulation of the cell cycle–negative regulator p21 and the apoptosis-associated protease caspase 3 in tumor cells, which block tumor growth by induction of apoptotic cell death and G0/G1 phase arrest [54]. Data for the putative inhibitory effect of MSCs on lung carcinoma cells are conflicting. On the one hand, Li et al. demonstrated that MSCs inhibit the proliferation of SK-MES-1 and A549 lung cancer cells both in vitro and in vivo [55]. In contrast, another work of the same group reported that MSCs play a dual role on the growth of lung carcinoma cells in vitro and in vivo, namely inhibiting A549 lung carcinoma cell proliferation in vitro, but propagating tumor formation and growth in vivo [56].

Even though it seems to be a little bit curious that one group presented data demonstrating that MSCs either block or propagate tumor growth in vivo, these findings point to the pro-tumorigenic effect of MSCs. In this context, a landmark study published by Karnoub et al. indicated that MSCs within tumor stroma promote breast cancer metastasis [43]. Thereby, breast cancer cells stimulate the de novo secretion of CCL5/RANTES from mMSCs, which then act in a paracrine fashion to enhance their motility, invasion, and metastasis [43]. Inhibition of CCL5/RANTES-CCR5 signaling, either by siRNA-mediated knockdown of CCR5 or by addition of an anti-CCL5/RANTES antibody, markedly reduced the metastatic spreading of breast cancer cells [43], clearly indicating the supportive effect of MSCs in promoting breast cancer metastasis.

This positive feedback loop between MSCs and breast cancer cells resembles a similar mechanism that has been described for the interplay of macrophages and breast cancer cells. Here, macrophages express EGF, which promotes the expression of colony stimulating factor-1 (CSF-1) and cell invasion of breast carcinoma cells. CSF-1 promotes the expression of EGF in macrophages, which in turn promotes the expression of CSF-1 by breast carcinoma cells, thereby generating a positive feedback loop [57]. Data of Rhodes et al. indicate that MSCs could contribute to primary breast cancer tumor growth and the progression of these tumors to hormone independence [58]. Co-injection of BM-MSCs with estrogen-receptor (ER) positive, hormone-dependent MCF-7 breast cancer cells enhanced primary tumor growth in an animal model irrespective of the presence of absence of estrogen [58]. Moreover, the authors were able to demonstrate the hormone-

independent growth of MCF-7 cells when co-injected with BM-MSCs [58]. Immunohistochemistry revealed increased progesterone receptor levels in MCF-7/BM-MSC tumors, which may indicate a link between MCF-7 cells and BM-MSCs through ER-mediated signaling [58]. Further data of Rhodes et al. revealed increased SDF-1α levels both in vitro and in vivo when MCF-7 breast cancer cells were co-cultured with BM-MSCs [59].

Because SDF-1α is an ER-mediated gene linked with hormone-independence and metastasis, BM-MSCs could promote MCF-7 tumor growth by secretion of SDF-1α in an estrogen-dependent manner [59]. In addition to primary tumor growth, MSCs may further positively trigger the survival of breast cancer-initiating cells (BCICs) within the bone marrow [60]. Even though it is not quite clear how MSCs support BCICs, these data indicate that MSCs do not only promote the initial step in cancer metastasis—namely, the aforementioned EMT—but most likely also favor the reverse process (mesenchymal-epithelial-transition) once the metastatic cancer cells have found their destined tissue(s).

A study by Comsa et al. indicated that MSCs can further promote breast tumor growth by adopting a vascular phenotype [61]. Both MCF-7 breast cancer cells and VEGF stimulated MSCs to form capillary-like structures, indicating a role of tumor-derived VEGF in modulating their recruitment into sites of pathological vasculogenesis [61].

In addition to breast cancer, a pro-tumorigenic effect of MSCs has further been described for osteosarcoma [62, 63]. Xu et al. demonstrated that MSCs are recruited to the osteocarcinoma site in a SDF-1α dependent manner and that MSCs promote osteocarcinoma cell migration by secretion of CCL5/RANTES [62], which is similar to breast cancer. Likewise, MSCs might promote tumor engraftment and metastatic colonization by providing a suitable microenvironment for metastatic osteosarcoma cells [63]. Interestingly, MSCs may not only promote osteosarcoma progression, but might be themselves the source of osteosarcomas [64]. Long-term cultivation of MSCs was associated with aneuploidization, translocation, and homozygous loss of Cdkn2, representing the key mediators of MSC malignant transformation [64]. Because CDKN2A/p16 protein expression in 88 patients with osteosarcoma was identified as a sensitive prognostic factor, the authors concluded that this could bridge the murine MSC model to human osteosarcoma [64].

Several lines of evidence indicate that MSCs might harbor an oncogenic capacity when cultured for long periods. Li et al. demonstrated that aged MSCs can spontaneously transform in culture and, when inoculated in mice, recapitulated the naturally occurring fibrosarcomas of the aged mice with gene expression changes and p53 mutation [65]. Moreover, spontaneously transformed MSCs contributed directly to the tumor, tumor vasculature, and tumor adipose tissue, as well as recruited additional host BM-derived cells to the area, which fused with transformed MSCs [65]. Interestingly, such hybrid cells exhibited a rather nonmalignant phenotype, whereas unfused transformed acted as cancer stem cells [65]. This finding is opposed to the hypothesis that cell fusion between tumor cells and normal cells could give rise to hybrid cells exhibiting novel properties, such as an enhanced metastatogenic capacity or drug resistance, thus contributing to tumor progression

[66–68]. In any case, published data revealed that the fusion of human umbilical cord MSCs with esophageal carcinoma cells can inhibit the cancer cells' tumorigenicity, most likely due to induction of apoptosis [69].

Implantation of MSCs, which were genetically modified by the nonviral Sleeping Beauty transposon assay to stably express firefly luciferase and dsRed fluorescent protein, into mice resulted in development of foci of sarcoma in all animals; this suggests that MSCs have undergone malignant transformation during cultivation [70]. In fact, original, nontransfected MSCs cultures were found to be cytogenetically abnormal; primary MSCs derived from both BALB/c and C57BL/6 mice showed cytogenetic aberrations after several passages in vitro [70]. Thus, long-term cultivation rather than genetic modification of MSCs might have been the reason for malignant transformation. This agrees with data from Foudah et al. who monitored the genomic stability of in vitro cultured rat BM-MSCs [71]. By combining traditional cytogenetic techniques and comparative genomic hybridization, it was shown that rat BM-MSCs manifested a markedly aneuploid karyotype and a progressive chromosomal instability irrespective of culture conditions, indicating that they are anything but stable during in vitro culture [71]. Interestingly, another study revealed that MSCs might undergo chromosomal abnormalities even at early passages [72]. In this work, MSCs of passage 4 were transplanted to study their therapeutic effects in animal models of experimental myocardial infarction and diabetic neuropathy [72]. During the follow-up at 4–8 weeks later, growing tumors were observed in 30 of hearts in the myocardial infarction model and in 46 % of hindlimbs in the diabetic neuropathy model [72]. Characterization of tumor samples revealed hypercellularity, pleomorphic nucleoli, cytological atypia, and necrosis, as well as multiple chromosomal aberrations including fusion, fragmentation, and ring formation [72]. Similar data were provided by Grimm et al. demonstrating that periodontium-derived stem cells (pdSCs), which are of ecto-mesenchymal origin, could induce tumor growth in an athymic rat model [73]. Pathological and immunohistochemical analysis demonstrated that tumors could be identified as a kind of anaplastic squamous epithelial-cell carcinoma with human mitochondria in rat tumor tissue, indicating that the tumor most likely originated from implanted pdSCs [73]. Here, pdSCs were grown as dentospheres and were expanded for only up to 10 days, indicating a short-term cultivation period [73]. Karyotypic analysis of expanded pdSCs showed a high degree of aneuploidy, with chromosome counts peaking at 70 chromosomes [73].

In contrast to the previous summarized studies, several studies provided evidence that MSCs neither undergo malignant transformation after long-term in vitro culture nor exhibit telomere maintenance mechanisms [74]. Bernardo et al. studied BM-MSCs from 10 healthy donors that were propagated in vitro until reaching either senescence or passage 25, which were subsequently analyzed thoroughly by array-comparative genomic hybridization (array-CGH), conventional karyotyping, and subtelomeric fluorescent in situ hybridization analysis as well as the expression of telomerase activity, human telomerase reverse transcriptase (hTERT) transcripts, and alternative lengthening of telomere (ALT) mechanism [74]. Data revealed a

huge variability in terms of proliferative capacity and MSC lifespan among donors, but array-CGH and cytogenetic analyses showed that in vitro expanded MSCs did not show any chromosomal abnormalities [74]. Likewise, telomerase activity and hTERT transcripts were not expressed and prolonged cultivation of MSCs was associated with telomere shortening, indicating that MSCs can be safely expanded in vitro [74]. Similar data were provided by Choumerianou et al. [75], Poloni et al. [76], and Mareschi et al. [77]. All of these studies demonstrated that MSCs could be propagated in vitro without malignant transformation. Thus, the data on whether MSC cultivation is associated with malignant transformation remain conflicting. Nonetheless, this knowledge is of crucial interest for the use of MSCs in regenerative medicine or anti-tumor strategies.

In addition to stimulating tumor progression by interacting with tumor cells or by being themselves tumorigenic, MSCs can further foster tumor growth by their immune-modulating capacities. It is well recognized that MSCs can interact with cells of the innate and the adaptive immune system as well as induce peripheral tolerance [21]. In the context of cancer, the immunosuppressive capacity concomitant with the tissue regeneration properties of MSCs are of interest; they resemble conditions mediated by M2-polarized macrophages [78–80]. In brief, MSCs favor tumor growth because of immune suppression and secretion of growth factors. This topic has been recently summarized in an excellent review by Uccelli et al. the reader is referred to this paper on how MSCs trigger the activity of B- and T-lymphocytes, NK cells, monocytes, and dendritic cells [21].

2.3 MSCs as Trojan Horses for Cancer Therapies

Because of tumor tropism, MSCs have been designated as ideal cellular vehicles for anti-cancer therapies. Like Trojan horses, genetically modified MSCs defeat tumor and metastatic growth from inside the cancerous tissues. In this section, we summarize the current concepts on how MSCs are genetically modified to achieve optimal anti-tumorigenic properties.

2.3.1 MSCs as Cytokine-Producing Cells

A landmark study by Studeny et al. demonstrated the feasibility of MSCs acting as cytokine-producing cells that infiltrate tumor tissue and inhibit malignant cell proliferation from the inside [81]. Thereby, inhibition of A375SM melanoma cell growth in vivo mediated by interferon-β (IFN-β) producing MSCs required MSC tumor integration and was not achieved by systemically delivered IFN-β or IFN-β produced by MSCs at a site distant from the tumors [81]. These data indicate the necessity of MSCs migrating into cancerous tissue to exert their anti-tumorigenic properties. In addition to melanoma, further in vivo studies revealed that IFN-β secreting MSCs also effectively inhibit the growth of MDA-MB-231 and 4T1 breast

cancer cells [44, 82], U87 glioma cells [83], and T24 N bladder carcinoma cells [84]. Studies by Ren et al. indicated that IFN-β producing MSCs significantly reduced the tumor volume in a mouse model of prostate cancer and lung metastasis [50], indicating that not only primary tumors but also metastases are effectively targeted by such an approach. The knowledge that MSCs are even capable of killing metastases efficiently is of crucial interest in cancer therapy because the primary tumor is generally surgically removed and thus does not need to be treated. However, metastases are still a major problem in cancer and cancer therapy. It is well recognized that more than 90 % of patients with cancer do not suffer from the primary tumor but rather from its metastases. In fact, therapy options once metastases have formed are limited and are generally rather palliative than curative. Because of that, the data from Ren et al. and others (see below) are encouraging.

In addition to IFN-β, interleukin-2 (IL-2) and interleukin-12 (IL-12) have been chosen as other cytokines for MSC-based anti-cancer approaches. Co-injection of IL-2 producing MSCs with B16 cells significantly delayed tumor growth by inducing CD8- and NK cell-mediated anti-tumor activity [85]. Likewise, IL-2 producing MSCs augmented the basal MSC anti-tumor effect and prolonged the survival of 9L glioma-bearing rats [86]. Application of IL-12 producing MSCs strongly reduced the formation of lung metastases of B16F10 melanoma cells, concomitant with a prolonged survival of animals in a mouse melanoma model [87]. In accordance with IL-2 producing MSCs, the anti-tumor activity of IL-12 expressing MSCs depended on NK cells and CD8 T cells [87]. Similar data were provided by Chen et al., who studied the anti-tumor and anti-metastatic capacity of IL-12 secreting MSCs in an advanced and pre-established metastatic mouse model using B16 melanoma cell, 4T1 breast tumor cells, and Hca hepatoma cells [88]. Here, progression of metastases into multistep lymph nodes and internal organs was markedly impeded and even reversed in the ultimate stage following a 20-day course of immunotherapy with IL-12 producing MSCs, whereby cells were administered once every 5 days [88]. The therapy was without systemic toxic effects and histomorphometry analysis revealed a reductive tendency towards reversion of tumor-associated lymphatic sprouts and an increased tumor apoptosis index in animals treated with IL-12 producing MSCs [88]. These data further support the potential use of genetically modified MSCs in treating cancer metastases. In addition to triggering NK cell and CD8 T cell activity, data from Eliopoulos et al. indicated that IL-12 producing MSCs also exhibited anti-angiogenetic capacities [89]. In this study, fewer tumor cells were detected in implants of 4T1 breast tumor cells with IL-12 producing MSCs concomitant with the presence of necrotic tumor islets and necrotic capillaries [89]. This anti-angiogenetic effect most likely depended on the IL-12 dependent induction of interferon-γ (IFN-γ) and the IFN-γ dependent induction of interferon-inducible protein 10 (IP-10) [90]. Compared to IFN-γ, IP-10 is a much more effective inhibitor of angiogenesis and in vivo data provided evidence that this factor is a mediator of tumor necrosis [91]. Elevated IFN-γ levels concomitant with anti-angiogenesis and increased T cell infiltration, which inhibited tumor growth, were also observed in a gene therapeutic approach of intracranial glioma using IL-12 expressing MSCs [92]. Interestingly, IL-12 producing MSC–treated and tumor-free

animals were resistant to ipsilateral and contralateral tumor rechallenge that was closely associated with tumor-specific long-term *T* cell immunity [92]. These data may point to another aspect of MSC-based anti-cancer strategies—namely, not only selectively defeating cancer cells but also inducing a putatively long-lived immunity against a particular tumor type, thereby minimizing the risk of cancer recurrences.

2.3.2 MSCs as Inducers of Apoptosis

Several studies demonstrated that MSCs exhibit increased anti-tumorigenic properties when expressing tumor necrosis factor–related apoptosis inducing ligand (TRAIL) [40, 46, 49]. Sasportas et al. showed that TRAIL-expressing MSCs induce caspase-mediated apoptosis in established glioma cell lines as well as CD133-positive primary glioma cells in vitro and have profound anti-tumor effects in vivo [46]. Particularly, the anti-tumorigenic effect of TRAIL-expressing MSCs seems interesting because CD133 has been suggested as a brain tumor stem cell marker [93, 94]. Current anti-cancer concepts favor the eradication of cancer stem cells because only the elimination of the tumor initiating cells will ensure the definite cure of cancer [67, 95–98]. Data from Kim et al. revealed that irradiation enhances the tumor tropism and therapeutic potential of TRAIL-expressing MSCs, which is most likely attributed to the irradiation-mediated tissue destruction and thus induced inflammatory conditions [40]. As summarized previously, MSCs are recruited to sites of inflammation to regenerate degenerated tissue [16–22]. Even though irradiated glioma cells showed increased IL-8 expression causing CXCR1/CXCR2 upregulation in MSCs [40], it can be assumed that further pro-inflammatory cytokines/chemokines might be upregulated as a consequence of irradiation, which will additionally trigger the recruitment of MSCs. Nonetheless, enhancement of the tumor tropism of MSCs (e.g., by irradiation) might be a therapy option for certain tumors to boost the anti-tumorigenic efficacy of applied genetically modified MSCs. In accordance to cytokine-secreting MSCs, TRAIL-expressing MSCs also exhibit an anti-metastatic capacity [49]. Compared to control animals, systemically delivered TRAIL-expressing MSCs specifically localized to lung metastases and completely cleared the metastatic disease in 38 % of mice [49], indicating the potent anti-metastatic capacity of TRAIL-producing MSCs.

2.3.3 Genetic Modification of MSCs

The previously summarized data revealed the anti-tumorigenic and anti-metastatic properties of genetically modified MSCs, either by expressing anti-tumorigenic cytokines or apoptosis-inducing factors, which seems encouraging for future MSC-based anti-cancer strategies. However, each of the currently used techniques for MSC gene transduction has limitations and may even have risks. Efficient transduction of cells and stable transgene expression is generally achieved with viral vectors, including adenoviruses, adeno-associated viruses, and lentiviruses. The use

of adenoviruses for gene therapeutic approaches is generally limited because they are highly immunogenic and the genome is not integrated in the host [99]. Although this will prevent insertional oncogenesis, the transgene is lost in diving cells [99]. However, these limitations (immunogenic and non-DNA insertion) have been overcome successfully in recent years by developing custom-made and improved adenoviruses that exhibit less immunogenicity, concomitant with the capacity to integrate the "transgene-of-interest DNA" in the host genome [100].

One strategy to modify adenoviruses to host DNA integration is by using naturally occurring DNA integration-promoting elements or sequences of the seemingly non-pathogenic helper-dependent parvovirus adeno-associated virus (AAV) [101]. The advantage of AAVs is that they preferentially integrate in a non-random manner into the so-called AAVS1 locus on human chromosome 19 [102], thus avoiding insertional oncogenesis. The current state-of-the-art technique of eukaryotic cell transduction is the use of lentiviruses, which are a subclass of retroviruses, and which efficiently integrate their genome in both dividing and non-dividing cells, thereby ensuring stable transgene expression [103]. The use of lentiviruses does carry a risk of insertional oncogenesis. However, hot spots of retroviral integration have been identified in human CD34$^+$ hematopoietic cells [104], indicating that the viral genome is not as randomly integrated as originally supposed. Likewise, data from Montini et al. provided evidence that tumorigenesis in a tumor-prone mouse model was unaffected by lentiviral vectors [105]. Accordingly, lentiviral transduction of MSCs neither altered DNA copy number nor resulted in chromosomal rearrangements [46]. Nonetheless, retroviral vectors still possess the capacity of insertional oncogenesis, which may limit their use in human therapies. Whether the use of human artificial chromosomes (HACs) might be a suitable alternative to viral vectors for genetic modification of MSCs remains to be elucidated in further studies. Data from Kinoshita et al. revealed that MSCs bearing a HAC vector containing the herpes simplex virus thymidine kinase gene migrated towards malignant melanoma in vivo and treatment with ganciclovir significantly reduced the tumor mass in an animal setting [106]. HACs might be advantageous for gene therapeutic approaches, including genetic modification of MSCs, because they are nonintegrating but self-replicating vectors with no limitations on the size and number of genes that can be inserted [106].

2.3.4 MSCs as Carriers for Oncolytic Viruses

The use of oncolytic viruses is another therapy option that has been developed for treating cancer. Wild-type viruses or genetically modified viruses can selectively replicate and kill tumor cells, whereas normal cells are spared. In this context, landmark studies were already published about 15 years ago by Frank McCormick and his team, who demonstrated the feasibility of a mutant adenovirus that selectively replicates and eliminates p53 negative human tumor cells [107]. However, as mentioned previously, adenoviruses are highly immunogenic, which is a limiting factor because this impedes or even precludes repeated rounds of treatment. Thus,

adenoviral vectors have been (and are still) designed and improved to decrease their immunogenicity as well as to enhance their tumor recognition specificities and tumor replicative capacities. Additionally, cellular vehicles are used for viral delivery to the tumor sites and MSCs appear to be the most attractive candidates for this purpose [108–110]. The feasibility of this approach was already demonstrated for breast cancer [108, 110], ovarian cancer [111], and intracranial gliomas [109, 112]. Moreover, the initial clinical trials are promising. For instance, infusion of autologous MSCs infected with the new oncolytic adenovirus ICOVIR-5 in four children with metastatic neuroblastoma demonstrated that the tolerance of the treatment was excellent [113]. Likewise, a complete clinical response was documented in one case and the child was in complete remission 3 years after this therapy [113].

3 Conclusion

The distinct tumor tropism of MSCs make these cells suitable candidates for anti-tumor and even anti-metastatic strategies, either due to their putative anti-tumorigenic capacity; the genetic modification and expression of transgenes, including IFN-β, IL-2, IL-12, or TRAIL; or as cellular vehicles for the delivery of oncolytic viruses. However, even though most of the data, particularly the in vivo findings, seem very promising, several pitfalls currently limit the use of MSCs in anti-cancer strategies, particularly the conflicting data on the potential oncogenic risk of MSCs in long-term cultivation. Because genetic modification demands cultivation of the cells for a defined period of time—and even short-term cultivation of MSCs may be associated with malignant transformation [72]—this issue has to be resolved first. At minimum, a thorough characterization of cultivated MSCs should occur prior to reapplication. In addition, the use of retroviral vectors for transgene expression is a potential risk due to the random insertion of the viral DNA into the host genome; this issue also must be resolved prior to the safe use of genetically modified MSCs. Nonetheless, MSCs are promising cellular vehicles that will be helpful in future anti-cancer strategies by delivering biotherapeutics (cytokines, apoptosis inducing factors, oncolytic viruses, etc.) directly into the tumor and its metastases.

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Dissecting Paracrine Effectors for Mesenchymal Stem Cells

Stefania Bruno, Federica Collino, Ciro Tetta and Giovanni Camussi

Abstract There has been increasing interest in the application of mesenchymal stem cells (MSCs) in regenerative medicine in recent years. In this context, the beneficial effects of MSCs have been ascribed mainly to a paracrine action rather than to direct replacement of the injured tissue. Indeed, MSCs produce a great variety of trophic and immunomodulatory factors. In this chapter, we provide an overview of growth factors and chemokines involved in stimulation of cell proliferation, inhibition of apoptosis, enhancement of angiogenesis, and suppression of inflammatory and immune response. In addition, we discuss the emerging role of the extracellular vesicles released from MSCs as possible paracrine mediators.

Abbreviations

AKI	Acute kidney injury
Akt-MSCs	Mesenchymal stem cells overexpressing the Akt gene
ALI	Acute lung injury
Ang-1	Angiopoietin-1
bFGF	Basic fibroblast growth factor
CM	Conditioned medium
HGF	Hepatocyte growth factor
IDO	Indoleamine 2,3-deoxygenase
IGF-1	Insulin-like growth factor 1

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IL-6	Interleukin-6
KGF	Keratinocyte growth factor
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MV	Microvesicle
NK	Natural killer
PGE2	Prostaglandin E_2
siRNA	Small interfering RNA
TGF-β	Transforming growth factor β
T_{reg}	Regulatory T cells
VEGF	Vascular endothelial growth factor

Contents

1 Introduction

Mesenchymal stem cells (MSCs) play an important supportive role in the bone marrow microenvironment, only partly mediated by cell-to-cell contact. Increasing evidence indicates that the biological effects of MSCs largely depend on paracrine mechanisms, involving bioactive factors active on neighboring cells. MSCs localize in perivascular areas in the bone marrow in close association with hematopoietic stem cells [78] and contribute to the quiescence of these cells by inhibiting both proliferation and differentiation [31]. Consistent with this purported role are the highly expressed transcripts of maintenance factors for hematopoietic stem cells, including the chemokine CXCL12, stem cell factor, angiopoietin-1 (Ang-1), interleukin-7, vascular cell adhesion molecule 1, and osteopontin [58].

MSCs may have potential therapeutic application in acute tissue injury of different organs (heart, kidney, lung, and liver), and they are currently used in clinical trials for treating a wide range of diseases (http://www.clinicaltrials.gov). Although MSCs exhibit multilineage differentiation potential and migrate to injured sites after systemic administration, recent studies have suggested that the differentiation of
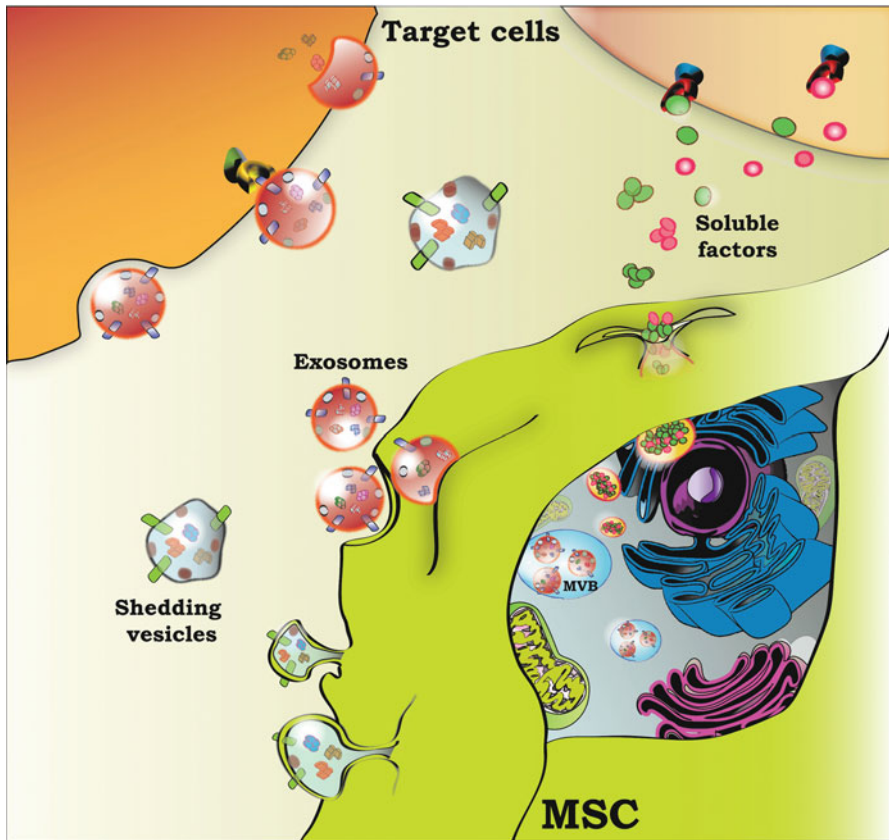
Fig. 1 Mesenchymal stem cells (MSCs) release bioactive factors mediating cell-to-cell communication. MSCs exert effects on surrounding cells, primarily through the release of paracrine effectors. These may include soluble factors or extracellular vesicles. MSCs synthesize and secrete a broad spectrum of soluble factors (listed in Table 1) responsible for MSC regenerative and protective functions. Soluble molecules are secreted from MSCs after fusion of secretory granules with the plasma membrane. Their effects are mediated via membrane receptor interaction with recipient cells. MSCs may also communicate through microvesicles (MVs). On the basis of the mechanism of formation, MVs may be distinguished into shedding vesicles or exosomes. Exosomes derive from the endosomal membrane compartment and are released after fusion of multivesicular bodies (MVB) with the plasma membrane of the donor cells. Shedding vesicles are generated by direct budding from the plasma membrane with formation of protrusions that detach from the cell surface. Once released from MSCs, MVs interact through specific receptor–ligand interactions with the recipient cells and fuse directly with their plasma membrane or are endocytosed and then fuse with an endocytic compartment of the cell. MVs may deliver surface receptors, proteins, bioactive lipids, and genetic material (messenger RNAs and miRNAs) to recipient cells, altering their functions

MSCs in cells of the injured tissues contributes little to the therapeutic benefit. A bulk of evidence indicates that the therapeutic effect of MSCs depends primarily on their capacity to secrete soluble factors (Fig. 1) having several biological activities:

inhibition of fibrosis and apoptosis, proangiogenesis, stimulation of proliferation and/or differentiation of tissue-intrinsic reparative progenitor cells [11], and immunomodulation [90]. MSC-secreted bioactive molecules act by direct signaling activation in target cells and/or indirectly causing neighboring cells to secrete functionally active agents [11].

Recent studies have shown that beside soluble factors, small vesicles released from cells, named extracellular vesicles or microvesicles (MVs), are also instrumental in cell-to-cell communication [10, 70, 71] (Fig. 1). It has been shown that MVs from MSCs contribute to tissue repair in different animal models of tissue injury [9, 29, 49]. We will analyze the paracrine action of MSCs in different acute disease settings.

2 Cardiac Injury

In preclinical animal models, MSCs derived either from autologous or allogenic sources have been shown to improve perfusion, attenuate myocardial scarring, and restore cardiac function after myocardial infarction [3]. These effects were interpreted as not dependent on direct replacement of injured cells, but rather on paracrine effectors that facilitate endogenous repair processes [64, 77]. Indeed, MSCs may boost angiogenesis, stimulate endogenous myogenic cells, and stabilize the extracellular matrix by acting via both cell-to-cell interaction and soluble growth factor release [27, 83].

Gnecchi et al. [32, 33] showed that intramyocardial injection of MSCs overexpressing the Akt gene (Akt-MSCs) or conditioned medium (CM) from Akt-MSCs reduces infarct size in a rodent model of acute myocardial infarction to the same extent. This cardioprotective effect of the CM was attributed to hypoxia and to overexpression of *AKT*, which induce release of secreted frizzled related protein 2. Silencing secreted frizzled related protein 2 by the the small interfering RNA (siRNA) knockdown technique in Akt-MSCs was shown to abrogate the cytoprotective effect [59].

More recently, it has been shown that injection of MSCs or MSC-derived factors into the limb muscle away from the diseased heart caused cardiac repair and improved ventricular function through several mechanisms: increased cardiomyogenesis and angiogenesis and reduced myocardial apoptosis and fibrosis [76]. Extracardiac MSC administration in skeletal muscle provides proof that the cardiac repair can be achieved through trophic actions of MSCs independent of direct localization of stem cells in the myocardium.

Recently, Shabbir et al. reported a role for interleukin-6 (IL-6)-type cytokines in cardiac repair through the engagement of the skeletal muscle JAK–STAT3 axis. The IL-6 cytokine family, through the common glycoprotein 130 receptor [25], activates JAK–STAT3 signaling [17] in cultured myocytes and causes increased expression of the STAT3 target genes, hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF). These in vitro findings are corroborated by

in vivo studies showing that hamstrings into which MSCs had been injected exhibit activated JAK–STAT3 signaling and increase production of growth factors/cytokines [77]. In these experimental conditions, elevated growth factor levels are detectable not only at the site of injection but also in the liver and the brain, suggesting a global trophic effect. The host-tissue-derived factors, in turn, activate the endogenous cardiac repair by mechanisms dependent on Akt, extracellular-signal-regulated kinase, and JAK–STAT3. In fact, administration of the JAK–STAT3 inhibitor WP1066 abrogates the MSC-mediated host tissue growth factor expression and the functional cardiac improvement.

MSC transplantation attenuates myocardial fibrosis in a rat model of heart failure [54]. In this study, the authors demonstrated that CM from MSCs inhibits in vitro cardiac fibroblast proliferation and expression of collagen I and collagen III messenger RNA (mRNA). Indeed, MSCs express adrenomedullin, an antifibrotic factor, and, once transplanted, MSCs increase the expression of adrenomedullin in myocardium, suggesting that they may ameliorate myocardial fibrosis.

Kinnaird et al. showed that MSCs injected into the adductor muscles of the ischemic hindlimb significantly enhance perfusion and collateral remodeling of vessels, improving tissue injury. These beneficial effects occur without MSC incorporation within neoformed vessels [45]. CM from MSCs was shown to contain a number of growth factors, including VEGF, basic fibroblast growth factor (bFGF), placental growth factor, and monocyte chemoattractant protein 1. CM from MSCs enhances endothelial cell and smooth muscle cell proliferation [45]. Experiments based on blocking antibodies indicate that VEGF is involved mainly in endothelial cell proliferation, whereas bFGF is involved mainly in smooth muscle cell proliferation. In vivo, it has been shown that the levels of bFGF and VEGF increase in tissue into which MSCs have been injected and colocalize with MSCs [45].

3 Acute Kidney Injury

The role of MSCs derived from bone marrow in the recovery from kidney injury has been extensively investigated. Administration of heterologous MSCs accelerates recovery in acute kidney injury (AKI) induced by toxic agents [37, 38, 60, 61] or ischemia–reperfusion [22], and induces functional improvement in chronic kidney disease [14]. Several studies demonstrated that after systemic injection, MSCs accumulate at the site of injury. Some tubular engraftment has been observed in AKI induced by cisplatin [60, 61] and glycerol [37, 38]. However, as in the model of glycerol-induced AKI, most of the MSCs disappear from the kidney after few days [35]. In the ischemia–reperfusion injury model, permanent engraftment of MSCs in the kidney did not occur [22]. It has been suggested that MSCs do not replace renal tubular cells but mitigate injury by providing a paracrine support to the repair. With use of genetic fate-mapping techniques, it has

found that repopulation of tubules after AKI occurs from tubular cells that survived injury [40].

A paracrine role of MSCs in renal tissue repair has been further supported by experiments showing that CM from MSCs mimics the beneficial effects of the cells of origin [6]. Bi et al. suggested that homing is not an absolute requirement for MSC-based therapy, as the intraperitoneal administration of CM from MSCs to mice with cisplatin-induced AKI was found to be sufficient to diminish tubular cell apoptosis, to increase tubular cell survival, and to limit renal injury [6]. These data indicate that factors secreted by MSCs are responsible for the renoprotective effect, suggesting an endocrine/paracrine action. In particular, it has been demonstrated that MSCs can exert beneficial effects on tubular repair by producing mitogenic and prosurvival growth factors such as insulin-like growth factor 1 (IGF-1). Administration of IGF-1-gene-silenced MSCs in the murine model of cisplatin-induced AKI limits the protective effect of MSCs on renal function and tubular repair [42]. Moreover, Tögel et al. [82] reported that VEGF is also a critical factor in renal recovery: VEGF knockdown by siRNA reduces the effectiveness of MSC infusion in ischemia–reperfusion AKI.

4 Lung Injury

MSCs have been studied in several in vivo models of lung disease [34, 56, 62, 65, 66, 72, 88, 89, 91]. In bleomycin-induced lung injury and fibrosis, MSCs improve lung inflammation and survival when given intravenously. These effects are ascribed not to lung engraftment rates (less than 5 %) but rather to a paracrine mechanism [65, 66]. Ortiz et al. found that a subpopulation of murine and human MSCs (approximately 5 %) produces interleukin-1 receptor antagonist, which can attenuate the severity of bleomycin-induced lung injury.

Several studies have addressed the potential therapeutic effects of MSCs in ALI induced by intraperitoneal [88] or intratracheal [34, 89] administration of *Escherichia coli* endotoxin. Xu et al. [88, 89] found that intravenous administration of MSCs prevents the severity of this type of acute lung injury (ALI). MSCs produce several epithelial specific growth factors, including keratinocyte growth factor (KGF), also known as fibroblast growth factor 7. KGF reduces lung injury in mice models of pulmonary edema [52, 63]. In the ex vivo model of endotoxin-induced ALI in perfused human lung, the intrabronchial instillation of MSCs restores alveolar fluid clearance [52]. Experiments with blocking antibody indicate that the beneficial effect observed is, at least in part, due to the secretion of KGF from MSCs [52]. Several properties of KGF explain the therapeutic effect of human MSCs in restoring alveolar fluid clearance, including stimulation of alveolar epithelial type II cell proliferation and differentiation, production of surfactant

[52], inhibition of apoptosis [93], and stimulation of transcription and/or translation of the major sodium and chloride transport proteins [87].

The integrity of the lung microvascular endothelium is essential to reduce alveolar edema and prevent ALI. Paracrine soluble factors, such as Ang-1 and KGF, are potentially important in these effects. Human MSCs secrete a significant amount of Ang-1, which is a ligand for the endothelial Tie2 receptor and is a known endothelial survival and vascular stabilization factor [86]. Ang-1 reduces endothelial permeability and inhibits leukocyte–endothelium interaction by modifying the expression of endothelial cell adhesion molecules [48]. Moreover, it has been demonstrated that Ang-1 also has a beneficial effect on alveolar epithelial permeability to proteins [44]. Indeed, co-culturing of human MSCs restores normal type II cell epithelial permeability. Using siRNA knockdown of Ang-1, Fang et al. found that Ang-1 secretion is responsible for this effect by preventing actin stress fiber formation and claudin-18 disorganization through suppression of nuclear factor κB activity [26].

Another epithelial-specific factor secreted by MSCs is HGF, which was found to stabilize the integrity of pulmonary endothelial cells [7, 26].

5 Acute Liver Injury

Intravenous administration of CM from MSCs induces significant survival benefit in the treatment of fulminant hepatic failure. In a fulminant hepatic failure model induced by two injections of the hepatotoxin D-galactosamine, an intravenous bolus of CM from MSCs reverses organ failure in rats [67, 85]. Interestingly, from histological analysis, the striking reduction in leukocyte infiltration suggests that CM from MSCs may limit the inflammatory cascade by interfering with the function and the ability of immune cells to invade the injured tissue [67]. CM from MSCs contains a broad spectrum of molecules involved in immunomodulation but also in liver regeneration. Cluster analysis indicates that a large fraction (30 %) of CM from MSCs is composed of chemokines. After affinity chromatography, it has been found that the therapeutic activity of CM from MSCs is restricted to the heparin-binding fraction containing chemokines. In addition to a generic anti-inflammatory effect, CM from MSCs may exert a more direct antiapoptotic and prosurvival effect on hepatocytes [85]. Indeed, several of the molecules detected in CM from MSCs possess antiapoptotic and liver regeneration stimulating effects [67]. For example, VEGF is known to induce HGF secretion by stellate cells, which in turn induce the expression of hepatocyte mitogenic transforming growth factor β (TGF-β). HGF, present in CM from MSCs, is also known to inhibit apoptosis. IGF-1 and IL-6 are other MSC-secreted molecules with antiapoptotic effects in liver injury [39].

6 Immunomodulatory Properties of MSCs

The mechanism by which MSCs modulate the immune response is still under investigation, but it is evident that it involves both the release of soluble factors and the direct interaction of MSCs with dendritic or antigen-presenting cells.

MSCs may suppress several T-lymphocyte activities both in vitro and in vivo [1, 5, 20, 46, 57, 84]. MSCs alter the cytokine expression profile of dendritic cells, naïve and effector T cells, and natural killer (NK) cells to induce a more anti-inflammatory or tolerant phenotype and to increase the proportion of regulatory T cells (T_{reg} cells). As such, MSCs have been investigated as a new therapeutic strategy for graft-versus-host disease [51, 53] and Crohn's disease [28], and for the prevention of organ transplant rejection [12].

Prostaglandin E_2 (PGE2) is a lipid intermediate that is implicated in the immunomodulatory effects of MSCs. PGE2 is synthesized from arachidonic acid via the action of the constitutive cyclooxygenase-1 or the inducible cyclooxygenase-2 enzyme. Murine and human MSCs constitutively express cyclooxygenase-2. PGE2 production is upregulated after co-culture of human MSCs with peripheral blood mononuclear cells. Moreover, Aggarwal et al. demonstrated that human MSCs produce PGE2 in high concentration when co-cultured with T cells and that the inhibitors of PGE2 production diminish MSC-mediated immunomodulation in vitro [1].

Exposure to interferon-γ does not ablate MSC inhibition of T cell proliferation, but induces the expression of HGF and TGF-β_1 at concentrations that can suppress alloresponsiveness [73]. In addition, soluble factors such as nitric oxide can regulate the immunosuppressive effects of MSCs [74].

There is growing evidence that IDO, PGE2, and TGF-β_1 can represent relevant mediators of MSC inhibition of NK-cell functions [1, 20, 57]. IDO is the rate-limiting enzyme involved in the catabolism of the essential amino acid tryptophan and is also involved in the inhibition of T cell proliferation by dendritic cells [41]. MSCs do not constitutively express IDO, but when stimulated with interferon-γ, they can express this enzyme [57]. Spaggiari et al. [79] demonstrated the inhibition of NK-cell proliferation, when NK cells are cultured in the presence of MSCs; interestingly, blockade of both IDO and PGE2 completely restores NK-cell proliferation.

Dendritic cell maturation plays a key role in initiating T cell responses [55]. Recent studies indicate that MSCs disrupt the major functions that characterize the transition of dendritic cells from immature to mature stages, such as the upregulation of antigen presentation/costimulatory molecule expression, the ability to present a defined antigen, and the capacity to migrate in response to chemokine CCL19 [23]. Djouad et al. [21] reported that MSCs secrete IL-6, which is involved in the reversion of maturation of dendritic cells to a less mature phenotype. Chen et al. [13] demonstrated that blockade of PGE2 synthesis in MSCs reverses the inhibitory effects on dendritic cell differentiation and function. PGE2 and IL-6 can mediate the effects of MSCs on dendritic cells, thus leading to T cell suppression.

TGF-β_1 and PGE2 also have a relevant role in the generation and expansion of T_{reg} cells from CD4$^+$CD25$^-$ precursors [4, 92]. Cell contact, TGF-β_1, and PGE2 contribute to T_{reg}-cell induction by MSCs. Human MSCs also secrete the soluble MHC isoform of human leukocyte antigen G5 by a mechanism dependent on interleukin-10 and cell-to-cell contact [75]. Human leukocyte antigen G5 contributes to the expansion of CD4$^+$ CD25^{high}FOXP3$^+$ T_{reg} cells [75]. This observation may explain the sustained survival of T_{reg} cells and their suppressor phenotype over time [19]. The relevance of MSC induction of CD4$^+$ T_{reg} cells has also been studied in vivo. Pretransplant infusion of MSCs prolongs survival of a semiallogenic heart transplant through the generation of T_{reg} cells [12, 30, 69]. MSCs also induce other regulatory T cell populations, including CD8$^+$ regulatory cells [68].

7 Extracellular Vesicles Released from MSCs as an Emerging Paracrine Mechanism

Recent studies identified extracellular vesicles as a mediator of cell-to cell communication. The extracellular vesicles are formed by two distinct processes. They may derive from the endosomal membrane compartment: after fusion of multivesicular bodies with the plasma membrane of the donor cells, they are extruded from the cell surface as exosomes [36] (Fig. 1). Alternatively, extracellular vesicles may originate by direct budding from the cell plasma membrane as shedding vesicles [15] (Fig. 1). The vesicle population detectable both in vitro and in vivo is a mixture of exosomes and shedding vesicles and is collectively defined as MVs. It is now recognized that MVs may interact with cells through specific receptor–ligand interactions and after internalization in target cells they may transfer surface receptors, proteins, and bioactive lipids [80]. In addition, MVs contain selected patterns of mRNA and microRNA and may act as a vehicle for genetic exchange between cells [43, 2, 16, 18, 70, 71, 94]. Ratajczak et al. demonstrated that the MVs released from embryonic stem cells may reprogram hematopoietic progenitors by delivery of mRNA and proteins. We demonstrated that MVs generated from endothelial progenitor cells after internalization in normal endothelial cells activate an angiogenic program by a horizontal transfer of mRNA [18]. These results suggest that MVs may be important paracrine/endocrine mediators of signaling within stem cells and differentiated cells by transferring selected patterns of proteins, mRNA, and microRNA.

MSCs are an important source of MVs, and analyses of their nucleic acid content demonstrated the presence of mRNA representative of the multiple differentiative and functional properties of MSCs and of selected patterns of microRNA [9, 16].

We recently demonstrated that MVs derived from human MSCs stimulate in vitro proliferation and apoptosis resistance of renal tubular epithelial cells and

Table 1 The main bioactive factors present in conditioned medium from mesenchymal stem cells (MSCs) derived from bone marrow

Bioactive factors produced by MSCs	Brief description	Functions
Interleukin-6	Member of the interleukin-6 family that contains four conserved cysteine residues involved in two disulfide bonds	Cardiac repair Liver protection and regeneration Immunomodulation
Adrenomedullin	52 amino acid peptide, produced through cleavage of a 185 amino acid prohormone (pre-proadrenomedullin) Belongs to the calcitonin gene peptide superfamily	Ameliorates myocardial fibrosis
Vascular endothelial growth factor	Dimeric glycoprotein belonging to the cysteine-knot superfamily of growth factors	Cardiac repair Kidney regeneration
Basic fibroblast growth factor	Member of fibroblast growth factor family of heparin-binding proteins	Smooth muscle cell proliferation
Insulin-like growth factor 1	Hormone similar to insulin Consists of 70 amino acids in a single chain with three intramolecular disulfide bridges	Ameliorates acute kidney injury Liver protection and regeneration
Interleukin-1 receptor antagonist	Member of the interleukin-1 cytokine family Is a natural inhibitor of the proinflammatory effect of interleukin-1β	Attenuates the severity of bleomycin-induced lung injury and fibrosis
Keratinocyte growth factor	Also known as fibroblast growth factor 7 Is the 7th member of the fibroblast growth factor family	Reduces lung injury
Angiopoetin-1	Ligand for the endothelial Tie2 receptor	Prevents and reduces lung injury
Hepatocyte growth factor	Belongs to the plasminogen subfamily of S1 peptidases but has no detectable protease activity Is secreted as a single inactive polypeptide, is cleaved by serine proteases into a 69-kDa α chain and 34-kDa β chain	Reduces lung injury Liver regeneration Immunomodulation
Chemokines	Approximately 8–10 kDa in size Have 4 cysteine residues in conserved locations that are key to forming their 3-dimensional shape	Modulation of the inflammatory cascade Liver protection in fulminant liver failure
Prostaglandin E_2	A lipid intermediate synthesized from arachidonic acid via the actions of the constitutive cyclooxygenase-1 or the inducible cyclooxygenase-2 enzyme	Immunomodulation

(continued)

Table 1 (continued)

Bioactive factors produced by MSCs	Brief description	Functions
Transforming growth factor β_1	Polypeptide member of the transforming growth factor β superfamily of cytokines	Immunomodulation Hepatocyte mitogen
Indoleamine 2,3-deoxygenase	Rate-limiting enzyme involved in the catabolism of the essential amino acid tryptophan	Immunomodulation
Human leukocyte antigen G	Histocompatibility antigen	Immunomodulation
Extracellular vesicles: microvesicles and exosomes	Small vesicles released from cells containing bioactive lipids, proteins, messenger RNA, and microRNA	Cell-to-cell communication Kidney regeneration Cardiac repair

accelerate in vivo the functional and morphological recovery in severe combined immunodeficiency mice with glycerol-induced AKI [9]. When compared with MSCs, MVs were found to mimic the beneficial effects of the cells, suggesting that they may mediate several MSC regenerative functions. Since RNA inactivation in MVs reduced both their in vitro and their in vivo effects, a mechanism dependent on RNA delivery has been suggested. Indeed, the transfer of specific microRNA and mRNA and the translation into proteins in the recipient cells of mRNA shuttled by MVs has been shown both in vitro and in vivo [9, 16]. A similar protective effect of MVs derived from MSCs has been observed in a model of renal ischemia–reperfusion injury. In this model, the administration of MVs not only limits the acute injury by inhibiting apoptosis and stimulating proliferation, but also prevents the development of chronic renal disease [29].

Recently, it was reported that CM from MSCs derived from human embryonic stem cells significantly reduces infarct size in pig and mouse models of myocardial ischemia–reperfusion injury when administrated intravenously in a single bolus just before reperfusion [81]. The same authors further demonstrated, through size fractionation studies, that the active components of CM are large complexes of 50–200 nm [49, 50]. With use of electron microscopy, ultracentrifugation studies, mass spectrometry, and biochemical assays, these complexes have been identified as exosomes [49]. Therefore, the therapeutic activity of CM from MSCs derived from human embryonic stem cells has been attributed primarily to the exosomes.

8 Conclusions

The paracrine effects of MSCs introduce a different element to the therapeutic applications of MSCs in regenerative medicine. Even if in preliminary trials MSC-based therapy was found to be relatively safe, the administration of MSCs is not completely free of risks for patients. Indeed, occlusion of the distal microvasculature,

as a consequence of the relatively large cell size, may occur. In addition, experimental studies have shown possible maldifferentiation of engrafted MSCs, with calcification in the heart [8], differentiation into adipocytes in the kidney [47], and induction of fibroblast and myofibrobast proliferation in the lung [24]. If use of MSCs is replaced with administration of their bioactive factors (soluble paracrine factors or extracellular vesicles, see Table 1), many of the safety concerns and limitations associated with the transplantation of viable replicating cells could be mitigated.

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Proteomics Approaches in the Identification of Molecular Signatures of Mesenchymal Stem Cells

Yin Xiao and Jiezhong Chen

Abstract Mesenchymal stem cells (MSCs) are undifferentiated, multi-potent stem cells with the ability to renew. They can differentiate into many types of terminal cells, such as osteoblasts, chondrocytes, adipocytes, myocytes, and neurons. These cells have been applied in tissue engineering as the main cell type to regenerate new tissues. However, a number of issues remain concerning the use of MSCs, such as cell surface markers, the determining factors responsible for their differentiation to terminal cells, and the mechanisms whereby growth factors stimulate MSCs. In this chapter, we will discuss how proteomic techniques have contributed to our current knowledge and how they can be used to address issues currently facing MSC research. The application of proteomics has led to the identification of a special pattern of cell surface protein expression of MSCs. The technique has also contributed to the study of a regulatory network of MSC differentiation to terminal differentiated cells, including osteocytes, chondrocytes, adipocytes, neurons, cardiomyocytes, hepatocytes, and pancreatic islet cells. It has also helped elucidate mechanisms for growth factor–stimulated differentiation of MSCs. Proteomics can, however, not reveal the accurate role of a special pathway and must therefore be combined with other approaches for this purpose. A new generation of proteomic techniques have recently been developed, which will enable a more comprehensive study of MSCs.

Keywords Mesenchymal stem cells · Proteomics · Cell surface markers · Differentiation · Growth factors

Contents

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1 Introduction

Mesenchymal stem cells (MSCs) are undifferentiated, multipotent stem cells with ability to differentiate into many types of mature cells. MSCs can develop into distinct mesenchymal tissues, such as bone, cartilage, fat, tendon, muscle, and marrow stroma because all of these cell types originate from mesenchyme [1]. However, it has also been demonstrated that MSCs can be differentiated into non-mesodermal cell types, such as hepatocytes and neurons [2]. Consequently, MSCs have been applied for a range of tissue engineering applications, such as new bone formation, cardiovascular regeneration, and neuro-repair.

Tissue engineering constructs typically consist of a lattice-like or porous scaffold, stem cells, and bioactive molecules [3–5]. Of these components, MSCs can be differentiated to a wide range of cell types and also can promote the generation of blood vessels necessary for new tissue formation. MSCs have been isolated from nearly all connective tissues, such as fat, dental pulp, and umbilical cord. However, MSCs from bone marrow are, by far, the most studied given their natural healing ability; it appears these cells secret immunomodulatory factors, which inhibit possible rejection upon transplantation. Bone marrow MSCs

(BMSCs) are usually obtained through a small aspirate of bone marrow and cultured for the purpose of tissue engineering.

Although MSCs have been tested extensively in tissue regeneration, the regulation of MSC behavior is not well understood. Newly developed techniques such as proteomics, gene microarrays, and protein microarrays have been applied in the research of MSCs and produced valuable information. In this chapter, we will discuss the application of proteomics in the study of MSCs.

2 Present Problems in MSC Study

Despite the extensive research on MSCs for tissue engineering applications, a number of basic problems remain, which must be overcome before MSCs can be applied universally in the field.

2.1 Molecular Markers

The basic characteristics of MSCs are: (i) the ability to grow as adherent cells on tissue culture plastic; (ii) fibroblast-like morphology; forming colonies that support hemopoiesis; (iii) differentiation into cells of the mesodermal lineage, expressing stromal markers but not hematopoietic markers [6–9]. These cells are considered to be a heterogeneous population; the markers used to identify MSCs include CD29, CD44, CD73, CD166, CD90, and CD105 [10]. However, all of these markers are widely expressed in all stromal cell populations. As yet, there are no specific molecular markers that can be used for the isolation, purification, identification, and characterization of MSCs.

2.2 Determining Factors for MSC Differentiation to Terminal Mature Cells

Differentiation of MSCs to certain types of cells is determined by their microenvironment [11–13]. When these cells are grown in culture medium containing ascorbic acid, beta-glycerophosphate, and dexamethasone, MSCs develop into osteoblasts and osteocytes. In serum-free high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 ng/ml TGF-β3, 100 nM dexamethasone, 50 µg/ml ascorbic acid 2-phosphate, 100 µg/ml sodium pyruvate, 40 µg/ml proline, and a commercial preparation of ITS-plus (final concentration: 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenious acid, 5.33 µg linoleic acid, and 1.25 mg/ml bovine serum albumin), MSCs can be differentiated

to chondrocytes. In medium containing isobutyl-methylxanthine, indomethacin, insulin, dexamethasone, and cAMP, MSCs develop into adipocytes [12]. The molecular mechanisms governing how MSCs differentiate into different lineages still remain unclear.

2.3 Mechanisms for the Stimulation of MSCs by Growth Factors

The percentage of putative stem cells in BMSCs in whole bone marrow is less than 0.01 %; therefore, in vitro cell expansion is required to reach the necessary number of cells needed for tissue engineering [14]. However, this cell expansion comes at a cost: the multipotent functions of BMSCs rapidly decrease during in vitro culture. Approaches used to stimulate MSC proliferation and differentiation and maintain their long-time survival include bioactive materials, growth factors, hypoxic culture conditions, and the use of adenovirus gene delivery [15–17]. It has been shown that VEGF overexpression in MSCs increases homogenous vascularization in a scaffold [18]. There are no unified protocols for optimizing growth conditions for MSCs. The molecular mechanisms of growth factors to stimulate the capability of MSCs are still not fully understood.

3 Overview of Proteomics in MSCs

Proteomics is a technique that reveals differentially expressed proteins in two samples that have been subjected to different treatments [19]. It is a technical approach that has been used in many fields of cell biological research. It has been used extensively in the human genomic sequencing project and has revealed thousands of functional genes in humans. The purpose of this approach was to unravel the functions and protein–protein interaction of the expressed genes. Functional genomics was developed to reveal the function of genes in a high-throughput fashion. Microarray technologies, for example, allow the study of the expression of a vast number of genes in response to a given treatment condition. Similarly, the expression of protein-coding genes into proteins and their expression profiles can be investigated by high-throughput proteomics technologies. The biological processes of any organism involve a complex interaction of a network of proteins. In most cases, proteins interact with each other to both regulate and facilitate cellular processes. The traditional way to study these interactions is to focus on a few key proteins, which are then tested by experiments. The technologies that have now been developed make it possible to monitor a large number of gene and protein profiles, although there is still a limit as to how many proteins can be effectively monitored at any one time. At the gene level, a new generation of gene sequencing technology has made it possible to quickly sequence all genes of an organism. Proteins, on the other hand, are far more complex to investigate than

genes. This complexity stems from a number of factors. First, post-transcriptional alternative splicing of the primary mRNA can give rise to a number of protein isoforms, which are often expressed in a tissue-specific manner. In addition, almost all proteins undergo post-translational modification. Finally, the polarity, charge, and amenability of protein cleavage add another layer of complexity to the study of proteins.

Proteomics can reveal the altered expression of proteins by comparing samples subjected to different treatments. It can discover novel proteins involved in a biological process or how proteins respond to therapeutic agents to elucidate possible mechanisms. The chief limitation of proteomics is that it can detect only a proportion of the total protein output, typically highly abundant proteins. In yeast, 66 % of predicated open reading frames have been found by proteomics, whereas the figure for mycoplasma pneumonia is 60 %; in *Caenorhabditis elegans* and *Arabidopsis thaliana,* only 54 and 50 % have been found, respectively [20]. Nevertheless, the application of proteomics has produced valuable data to expand the knowledge base in a number of fields.

3.1 Protein Separation

In general terms, proteomics includes three steps: resolution of proteins followed by mass spectroscopy and bioinformatics analysis. The majority of proteomics studies use 2D gel separation and liquid chromatography/mass-spectrometry (LS/MS) [21, 22]. 2D-PAGE was developed in the 1970s and is still frequently used to separate proteins for further identification [23]. Proteins are separated on a polyacrylamide gel by gel electrophoresis based on size and isoelectric point. The first dimension separates proteins by their isoelectric points (pI) along with a pH gradient. The second dimension is molecular mass. After electrophoresis, the gel is stained to visualise the proteins. The appearance of protein spots are analysed by specialized software packages that can detect proteins that have changed as a result of the experimental condition. The limitations of 2D-PAGE are that it cannot separate proteins lighter than 10 kDa, larger than 150 kDa, or with a pI in the high basic range. Hydrophobic proteins, on the other hand, are not soluble and can therefore not even enter the gel; low-abundance proteins, such as growth factors, membrane, and signal transduction proteins, are undetectable by 2D-PAGE.

Differential gel electrophoresis (DIGE) can detect scarce proteins to as low as 150 pg and allows for the simultaneous separation of up to three samples in one gel by labelling the samples with different fluorescent dyes (CyDye 2, 3 and 5). However, the high cost of chemicals, instruments, and software limits the widespread application of DIGE as a proteomics platform.

In the past decade, proteomics has undergone rapid development. A new range of techniques have been developed in which HPLC based on hydrophobicity is used for protein separation and radioactive and fluorescent moieties are used to labelled the proteins in cultures or peptides produced by digestion. Protein

fractions produced by this method can then be analysed for protein expression by mass spectrometry. The application of this new suite of techniques to the study of MSCs should produce biological data that can to elucidate many of the knowledge gaps that exist with respect to the use of MSCs for clinical applications.

Newly developed proteomic techniques include isobaric tag for relative and absolute quantification (iTraq), Isotope-coded affinity tag (iCAT), global internal standard technology (GIST) and stable isotope labelling by amino acids in cell culture (SILAC). Of these, SILAC and iTraq are most commonly used. With SILAC, the proteins are labelled during cell culture with stable isotopes and then quantified by MS after gel separation. With iTraq, proteins are digested and the peptides produced are labelled by chemical isobaric tags and quantified by MS/MS [24]. iTraq can analyse up to eight samples in one run [25–28]. Individual samples are trypsinised and labelled with different kinds of iTraq reagents (114, 115, 116, and 117). These samples are mixed in equimolar concentrations and further separated by two-dimensional chromatography. This is a well-characterized technique that is used extensively in cancer research [29, 30].

3.2 Mass Spectrometry

Mass spectrometry is used to identify the proteins cut from spots of interest on a labelled gel. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) is most commonly used but has several disadvantages. It is not reliable for organisms that lack genomic information or for proteins with extensive cross homology. Furthermore, proteins with post-translational modifications cannot be detected and the technique fails to distinguish individual proteins in the one spot.

3.3 Bioinformatics Analysis

The final leg of proteomics is the application of bioinformatics software with which to analyse the data obtained from mass spectrometry. These software packages are usually used to classify proteins based on function and biological process and can quickly establish an initial analysis for the proteins with altered expression based on available databases from published literature. The commonly used software servers include Ingenuity pathway analysis (http://www.ingenuity.com), which is a fee-based service to life scientists, and the free services Panther (http://www.pantherdb.org) and DAVID (http://david.abcc.ncifcrf.gov).

3.4 Proteomics for Protein Modification Studies

Proteins are modified in many ways before they become fully functional; these modifications include phosphorylation, glycosylation, methylation, and ubiquitination. Traditional proteomics methods are unable to reveal such changes to the primary structure of proteins; therefore, special proteomic techniques have been developed for the purpose of studying these protein modifications.

Phosphorylation is a major post-translation modification of proteins, which changes protein function rapidly and reversibly. There are proteomics techniques that can characterise the phosphorylation status of proteins [31]. The majority of proteins are phosphorylated at multiple sites. Many antibodies are specifically designed to only recognise phosphorylated proteins; these are extensively used for studying signal transduction. Proteomics techniques, such as iTraq, can reveal novel phosphorylation sites that cannot be detected by available antibodies [32–34]. Such phosphorylation sites can subsequently be subjected to further study for new signal transduction pathways. Protein glycosylation is considered to be a good marker for diseases. For example, permeability glycoproteins (P-gp) have been studied extensively by proteomics [35–37]. P-gp are membrane transporters that play an important role in drug bioavailability, detoxification, and drug resistance in cancer [37].

Ubiquitination of proteins is a key regulatory process for the functioning of proteins during the life cycle of cells and refers to the covalent attachment of one or more ubiquitin molecules to a target protein, which leads to protein degradation and thus affects cell proliferation or death [38–40]. It has, for example, been shown to play a key role in the NF-kB signaling pathway by interacting with a proteasome complex, which processes the p105 precursor molecule into the active p50 domain [41, 42]. Ubiquitination proteomics has been specifically developed to study such post-translational modifications and has been used to screen all ubiquitination proteins after EGF induction; it has also been used in cancer and arthritis studies [43–48].

4 Application of Proteomics in the Study of Cell Surface Molecular Biomarkers of MSCs

Specific cell surface markers are important for the identification and purification of stem cells. It has, however, been difficult to find MCS-specific markers because these cell populations are heterogeneous in nature and cell surface markers are affected by culture condition. Proteomics has been used to identify specific markers on MSCs. To date, no specific signal molecules have been identified with MSCs but a specific pattern of protein expression has been shown to be typical of these cells. This pattern is positive for CD44, CD73, CD90, CD105, CD106 and CD166 and negative for CD31, CD34, CD45 and CD56 [49]. The identification of

this cell marker pattern is evidence of how proteomics has improved the identification of MSCs in very heterogeneous cell populations.

We have developed a system using single-cell clones (SCC) derived from bone marrow stromal cells (BMSCs) and characterised them by proteomics [50]. We isolated 14 clonal populations from three bone marrow stromal samples. The clonal populations were grouped into fast-growing clones and slow-growing clones by their ability to proliferate by measuring the time taken to reach 20 population doublings. Five of six fast-growing clones were able to differentiate into osteogenic, chondrogenic, and adipogenic lineages. The slow-growing clones showed limited differentiation and morphological changes. The markers CD29, CD44, CD90, CD105, and CD166 determined by flow cytometry were not different between the fast-growing and slow-growing clones [50].

Our study showed that eleven proteins were differentially expressed in clonal MSCs, including cytoskeletal and structural proteins, calcium binding proteins, cytokinetic proteins, and members of the intermediate filament family [51]. The proteins calmodulin (CALM), tropomyosin alpha-4 chain, and corticopin-lipoptotin were increased in fast-growing clonal SCC, whereas the expression of annexin 1, lamin A/C, progerin, caldesmon (CALD), heat shock protein 27, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase isozyme M1/M2, and enolase was increased in slow-growing clones [51].

CALM has been shown to inhibit apoptosis by binding and blocking Fas-mediated apoptosis and promote cell proliferation [52]. It also regulates osteoclastogenesis. CALM is a small Ca2 + binding protein and a central coordinator of many proteins involved in calcium metabolism. CALM has been demonstrated to interact with multiple intracellular targets [53]. It can bind to human epidermal growth factor receptor 2 (HER2), trigger a signaling cascade downstream from HER2, and promote cell growth [54]. HER2 is known to regulate fundamental cellular processes, including cell proliferation, migration, metabolism, and survival; it also has an important role in the differentiation of stem cells and plays a role in angiogenesis. Antagonist of HER2 has been shown to decrease neovascular formation via inhibition of hypoxia inducible factor (HIF) [52]. CALM interacts with EGFR, insulin receptor, and estrogen receptor alpha to activate PI3 K/Akt and MAPK pathways [55–57]. Our findings that CALM has an increased expression in fast-growing clones represent an explanation for this phenotypic trait.

Another protein identified to be overexpressed in a fast-growing clonal population is tropomyosin alpha-4 chain (TPM4). TPM4 is known to regulate the actin-cytoskeleton [57]. It forms rodlike polymers along grooves of actin filaments. TPM4 interacts with actin-binding proteins cofilin, gelsolin, Arp 2/3, myosin, and caldesmon and affects cell morphology. Mutations of the TPM4 protein cause cardiac and skeleton muscle diseases. Cytoskeleton dynamics is necessary for cell division and proliferation. It is, however, not known if the protein acts downstream from CALM. Our study has provided foundation for further elucidation of the mechanisms as to why some MSCs grow faster within cell populations.

5 Application of Proteomics in MSC Differentiation

The behaviours of BMSCs are largely affected by culture condition. Proteomics has been used to determine different protein expression profiles in MSCs when cultured in various defined media.

5.1 MSCs Differentiate to Osteogenesis

The differentiation of MSCs into osteoblasts in response to culture medium containing beta-glycerophosphate, dexamethasone, and ascorbic acid-2-phosphate has been well characterised. Growth factors in the medium can stimulate multiple signal pathways, which in turn regulate transcriptional factors for the expression of genes that control the formation of bone matrices and mineralization. Runx2 has been shown to play a central role in the process. Deficiency in Runx2 (heterozygous or duplications) caused cleidocranial dysplasia (CCD) and is responsible for about 10 % of CCD cases in humans [58]. In mouse models, homozygous mutation of the gene *Cbfa1*, which encodes Runx2, resulted in complete loss of bone formation and neonatal death. Heterozygous mutations caused specific skeletal abnormalities that show characteristics of the human heritable skeletal disorder, cleidocranial dysplasia (CCD) [59, 60]. The exact mechanisms that regulate the osteogenic response of BMSCs to the beta-glycerophosphate, dexamethasone, and ascorbic acid-2-phosphate containing media are still unclear.

A proteomics study identified PAI-1 (plasminogen activator inhibitor-1) as having an important role in osteogenesis [61]. This is consistent with the finding that bone formation increases in plasminogen-deficient mice. These studies suggest there is a plasminogen/PAI-1 axis that is important in osteogenesis. However, the proteomic study did not specifically target changes of other components in this pathway. Further studies of the PAI-1 pathway may reveal how MSCs differentiate into osteoblasts. Celebi et al. did a comprehensive proteomics analysis of MSC differentiation into different lineages [62]. A number of interesting targets were identified during osteogenic differentiation. One of these was 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, a rate-limiting enzyme in cholesterol biosynthesis and one that is downregulated in osteogenesis. This is an interesting finding because it is known that inhibition of HMG-CoA by statin resulted in increased bone formation [63]. BCAT (Branched chain amino acid aminotransferase) was also found to be strongly downregulated but its role in osteogenesis is not known.

Choi et al. have focused on the differential expression of extracellular matrix proteins using LC–MS/MS [64]. In conditioned medium collected from osteoblasts stimulated with osteogenic medium, they identified 64 differentially expressed

proteins. It was found that SPARC-related module calcium-binding protein 1 (SMOC1) was highly expressed. The importance of this protein was confirmed by both shRNA knockdown and protein overexpression. SMOC1 knockdown resulted in decreased mineralization, whereas its overexpression caused increased expression of the osteoblast differentiation markers ALP, COL1, OPN, SPARC, BGLAP, and IBSP.

It has been demonstrated that aging results in decreased population of MSCs in bone marrow; the evidence suggests that this is responsible for reduced osteogenesis and bone formation with aging. The in vitro ability of MSCs to differentiate to osteoblasts also decreases with increased time of population doublings. Proteomics has been used to unravel why the capacity of MSCs to differentiate into osteoblasts decreases with the number of passages [65]. In a serial subculture system, it was found that T-complex protein 1 subunit alpha (TCP-1) was gradually decreased with passage numbers and chloride intracellular channel 1 (CLIC1) expression fell in the early passages. These proteins could account for the reduced osteogenic potential of MSCs and warrant further studies. Increasing the number of available MSCs has been proposed as a means of treating age-related reduction of osteogenesis, but systemic infusion of MSCs has been shown to be ineffective due to the inability to MSCs to home to the bone. Therefore, genetic modification of MSCs has been used to increase the ability of MSCs to home to bone. It was reported that attaching the peptidomimetic ligand LLP2A to integrin $\alpha 4\beta 1$ on the MSC surface induced MSC migration and osteogenic differentiation both in vitro and in vivo [66].

5.2 MSCs Differentiate to Adipocytes

MSCs develop into adipocytes in adipogenic induction medium containing dexamethasone, methyl-isobutylxanthine, insulin and indomethacin. Proteomics has provided new insights into the mechanisms underlying this response to adipogenic medium. A study using 2DE and MALDI-TOF/MS identified several overexpressed proteins in this process, including syntaxin binding protein, oxysterol binding-3 related protein, phosphodiesterase PDE9A12, glycophorin, immunoglobulin kappa chain variable region, PPAR-gamma and T cell receptor V-beta 4 [67]. PPAR-gamma is a key regulator of adipogenesis; the fact that this protein is upregulated in MSCs in response to adipogenic induction media strongly suggests it is involved in the process of adipogenic differentiation of MSCs. The importance of PPAR-gamma in the generation of adipocytes was demonstrated in a study in which PPARγ1 and PPARγ2 were inhibited by BADGE and GW9662, as well as shRNA against PPARγ—all of which resulted in inhibited hMSC adipogenesis [68]. However, the inhibition of PPAR did not promote the osteogenesis

and expression level of the osteogenic transcription factor Runx2, indicating that other proteins expressed during adipogenesis put the brakes on osteogenesis.

5.3 MSCs Differentiate to Cardiomyocytes

Wakitani and Caplan were the first researchers to report that MSC could be induced to differentiate into cardiomyocytes by the action of the cytidine analogue isoprenaline (5-aza) [69]. MSCs treated with 5-aza for 24 h led to the formation of multinucleated myotubes 7–11 days later [70]. Interestingly, isoproterenol, which is commonly used for the treatment of slow heart rate, increased the beating rate of these cells—an effect that was blocked by beta-1-selective blocker CGP2072A [71]. In an in vivo model, implantation of MSCs into ischemic myocardium increased regional blood supply [72, 73]. Isoprenaline has been used extensively in animal models but its toxicity prevents it from being applied clinically. Alternative myocyte differentiating chemicals have therefore been tested.

The traditional Chinese medicine Shuanglong Formula (SLF) has been shown to have a cardiomyogenic effect on rat MSCs, similar to that of 5-aza and being far less cytotoxic. Proteomics was applied to determine the mechanisms for SLF-stimulated cardiomyocyte formation [74]. 2D gel analysis showed that the expression of 36 proteins were affected by SLF as well as by 5-aza. Of these proteins, 14 were downregulated and 21 were upregulated, and the expression of one protein was switched on. The increased proteins included vimentin, GAPDH protein, prolyl 4-hydroxylase, beta polypeptide (P4hb), peroxiredoxin 4 (Prdx4); the decreased proteins were actin, HSC70 polymerase (DNA directed) delta 1 catalytic subunit (Pold1), Pkm2 protein (Pkm2), S100 calcium binding protein A11 (S100a11), nucleoside diphosphate kinase B (Nme2), similar to ribosomal protein S12 (Rps12), eukaryotic translation elongation factor 2 (EEf-2), and fructose-bisphosphate aldolase A (ALDOA).

The majority of the proteins identified in this particular study are involved in the regulation of cytoskeleton, energy metabolism, and signal transduction. Of these proteins, vimentin changed the most and was regarded as a key protein in turning MSC into cardiomyocytes. Vimentin is a major cytoskeletal protein and is an organizer of a number of critical proteins involved in attachment, migration, and cell signaling [75]. Interestingly, an earlier study by the same group of authors showed that vimentin expression decreased rather than increased in response to 5-aza [76]. This discrepancy of results between two similar studies highlights the fact that proteomic studies can produce spurious results that need to be validated. In another proteomics study, the authors used porcine MSCs treated with 5-aza [77]. A total of 37 proteins were differentially expressed, of which 11 were upregulated and 26 were downregulated; B-crystallin, annexin A2 and stathmin 1 seemed to be important in the process induced by 5-aza, making it difficult to draw conclusions.

5.4 MSCs Differentiate to Neurons

MSCs from bone marrow aspirates have been differentiated into neurons and have potential uses for cell-based therapies for several neurological disorders, such as Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, multiple sclerosis, stroke, and spinal cord injury. It has been demonstrated that MSCs expressing nestin, a responsive marker, could differentiate into excitable neurons when co-cultured with cerebellar granule neurons [78]. Several signal molecules, including Sox2, Sox10, Pax6, Fzd, ErbB2, and ErbB4, were upregulated in these nestin-positive MSCs. 2D-DIGE analysis showed that 71 proteins were highly increased in nestin-positive cells, of which one-third were cell cycle regulators [79]. The authors also established clonal cells revealing that all nestin-positive cells express Sox10 and P75NTR, whereas nestin-negative controls were Sox10 negative and only weakly p75NTR positive.

5.5 Proteomic Profile of MSC Stimulated by Liver Differentiation Protocol

MSCs are also capable of differentiating into hepatocytes and therefore hold promise for liver-directed cell therapy [80]. A special protocol has been developed to turn MSCs into hepatocytes [81]. MSCs isolated from bone marrow are first cultured in DMEM supplemented with 20 ng/mL EGF and 10 ng/mL bFGF, after which the medium is replaced with one consisting of DMEM supplemented with 20 ng/mL HGF, 10 ng/mL bFGF, and 4.9 mmol/L nicotinamide. After seven days, the medium is changed to DMEM supplemented with 20 ng/mL OMS, 1 mmol/L dexamethasone, and 10 mL/mL ITS + premix (final concentration: 100 mmol/L insulin, 6.25 mg/mL transferrin, 3.6 mmol/L selenious acid, 1.25 mg/mL BSA, and 190 mmol/L linoleic acid) to achieve cell maturation, which is typically reached at day 21. The induction of hepatocytes-like cells was confirmed by Western blot analyses by assessing the expression of liver-specific markers. Two-dimensional gel electrophoresis and peptide mass fingerprinting MALDI- TOF-mass spectrometry showed that albumin, CK19 and CK20, FEM1B, PSMC2, and disulfide-isomerase A3 were significantly upregulated in MSCs treated with the liver differentiation protocol.

5.6 MSCs Differentiate to Pancreatic Islet Cells

MSCs have been induced to differentiate into pancreatic beta-cells that can secret insulin, thus opening the door for their potential uses in the treatment of diabetes. The growth medium used for this experiment was chondrogenic

differentiation medium with the addition of 10 ng/ml of TGF-beta3 [82]. The evidence of beta-cell activity was the presence of secreted insulin following the addition of glucose into the medium. Proteomics identified three important new proteins including apolipoprotein A-1 (APOA1), adult T cell leukemia protein 2 (ATL2), and superoxide dismutase (SOD2) [82]. Apolipoprotein A-1 is involved in lipid transport and metabolism in beta-cells and promotes in vitro insulin secretion in beta-cell cultures [83]. ALT2 is involved in endoplasmic reticulum membranes and its role in the pancreatic beta-cell differentiation is unknown; however, SOD2, which is found in mitochondria, protects beta cells from reactive oxygen species (ROS) damage, but its role in beta cell development remains unclear.

6 Application of Proteomics in the Effects of Growth Factors on MSCs

6.1 TGF-Beta Stimulation

Transforming growth factor beta 1 (TGF-beta 1) can act on MSCs via its cognate receptor to cause cell morphology change and an increase in actin fibers. Proteomic techniques were used to identify approximately 30 proteins with altered expression following stimulation with TGF-beta [84]. Besides upregulated or downregulated expression, the phosphorylation status of the proteins was also assayed. The proteins involved have a range of different functions, such as the cytoskeleton, matrix synthesis, membrane, and metabolic enzymes. Of the proteins identified by the proteomics assays, smooth muscle alpha-actin was increased, whereas the expression of gelsolin was decreased. These initial findings prompted the researchers to overexpress gelsolin, which led to inhibition of TGF-beta-induced assembly of smooth muscle alpha-actin. On the other hand, the knockdown of gelsolin expression enhanced the assembly of alpha-actin and actin filaments without affecting de novo alpha-actin expression. This study provides a good example of the utility of proteomics to flag putative pathways worthy of further study.

TGF-beta is a key regulator of cell growth, differentiation, migration, and extracellular matrix production. Interestingly, it can induce MSCs into either smooth muscle cells or chondrocytes, depending on cofactors in the culture medium [85–87]. Mechanical stimulation can synergise the effect of a growth factor [88]. Mechanical stimulation and TGF-β1 cause differential changes in MSC protein expression: the BGH3 gene has increased gene transcription, whereas CNN3 is upregulated after transcription.

6.2 Basic Fibroblast Growth Factor Stimulation

Basic fibroblast growth factor (bFGF) is commonly used for exogenous enrichment of MSCs [89]. It has been shown to increase the proliferative capacity of MSCs while maintaining their multilineage differentiation potential [90]. The receptors of bFGF, FGFR1/2 are expressed by mesenchymal progenitors in putative MSC niches in vivo, including the perichondrium, periosteum, and trabecular marrow. A comparative proteomics screen of membrane surface proteins of MSCs, treated with or without bFGF, identified 15 differentially expressed proteins in MSC cell surfaces [91]. This study could indicate the changes of markers in the cell membrane. However, the intracellular pathways were not shown. Characterization of intracellular pathways by proteomics may provide more valuable information. Studies have shown that FGFR activates several signal pathways, such as PKC, ERK and PI3K [92, 93].

The influence of bFGF on MSCs was investigated by a new proteomics technique called quantitative stable isotope labelling using amino acids in cell culture (SILAC). An analysis of cell secretomes was performed on MSCs transfected with bFGF to determine what factors were involved in an ectopic model of bone regeneration [94]. The transfected cells were shown to recruit host cells to form bone, whereas in the case of non-transfected controls, ectopic bone was only formed by the implanted cells. This has been associated with a subset of bFGF-stimulated MSCs, which were ALP negative and resulted in a higher frequency of colony forming unit–fibroblasts (CFU-f). A total of 67 proteins were found to be differentially expressed, of which the expression of 35 proteins were increased and 32 proteins were decreased. Gene ontology cluster analysis was performed using the functional annotation tool Database for Annotation, Visualization and Integrated Discovery (DAVID), which identified that bFGF-selected MSCs secreted a greater amount of proteins positively modifying the microenvironment and host cell activation; the most significant were involved in natural immune response, chemotaxis, inflammatory response, and response to wounding. The bFGF-selected MSCs secreted lower amounts of proteins related to processes involved in extracellular matrix organization; development of differentiated bone, skin, adipose, and blood vessels systems; response to wounding via complement activation and lymphocyte-mediated immunity. These findings provided evidence that bFGF-transfected MSCs recruit host cells to increase bone formation—a process critical for the repair of an injured tissue.

7 Application of Proteomics in Tissue-Derived MSCs

MSCs can be sourced from the sites other than bone marrow, which overcomes some of the disadvantages associated with relying solely on BMSCs for tissue engineering. Alternative sources of MSCs include adipose tissue, dental

tissue, pancreatic islets, placenta, and umbilical cord. Cells from these tissues are easily accessed and therefore overcome the relative difficulty of obtaining cells from bone marrow, which is an invasive procedure. BMSCs, as a fraction of the total stromal cell population, tend to become smaller with age and their proliferation and differentiation potential is rapidly reduced with passage numbers in vitro. Placental and umbilical cord MSCs can overcome some of the disadvantages of BMSCs by the fact that cells from placenta and umbilical cords are younger. In this section, our focus will be on proteomics studies that compare the properties of tissue-derived MSCs with BMSCs.

7.1 Adipose Tissue

Of all the potential sources of MCSs, adipose tissue is by far the easiest to obtain. MSCs have been isolated from adipose tissues and are shown to be capable of differentiating into terminal cells, such as hepatocytes and osteocytes. A study comparing MSCs isolated from bone marrow, adipose tissue, and amniotic fluids found that cells from all three sources could differentiate into hepatocytes-like cells, indicated by the hepatocyte markers ALB and TDO2 [80]. A study using DIGE proteomics showed that MSCs derived from fat were capable of expressing osteogenic markers when stimulated with osteogenic induction medium, containing dexamethasone, beta-glycerophosphate, and ascorbic acid [95]. Both cell types are positive for known mesenchymal markers such as CD44, CD73, CD90, and CD105 and negative for the haematopoietic markers CD31 and CD45. BMSCs express CD106 (VCAM1), an adhesion molecule found on vascular cells, but not CD34, which is usually detected on haematopoietic progenitors. By contrast, fat-derived MSCs are CD34 positive and CD106 negative. Proteomics has revealed that stromal cells from adipose tissue and bone marrow are fundamentally different cell types [96]. Markers specific for chondro/osteogenesis such as HES-1, DLX-5, TWIST1, osteocalcin, osterix, SOX9, WNT5A, TGFβ1, and VEGF were highly expressed in BMSCs compared to fat-derived MSCs. However, both cell types are equally capable of differentiating into chondrocytes and osteoblasts. These studies show that stem cells isolated from adipose tissue are multipotent and capable of differentiating into all the same terminal cell types as bone marrow stem cells, in response to the same induction media. There is clearly a role for proteomics to further characterise what pathways and proteins in the processes could performed.

7.2 Dental Tissue-Derived MSCs

MSCs have been identified in a number of dental tissues including follicle, pulp, and periodontal ligament by the expression of mesenchymal stem cell markers, such as STRO-1 and CD146 [97]. It has been proposed that these MSCs can be used for periodontal and bone regeneration [98, 99].

Dental follicle precursor cells are stems cells that are capable of differentiating into osteoblasts, cementoblast, and neuroblast and can form tooth root in a scaffold [100, 101]. The differentiation process of these cells has been studied and many signaling molecules are demonstrated to play important roles in the process, such as Wnt pathway and DXL3 [102, 103]. TPC (beta-tricalcium phosphate) can increase the differentiation of these cells into osteocytes [104]. Long-term stimulation of these cells by rhBMP-2 and/or rhBMP-7 significantly increased osteogenesis, as indicated by increased ALP activity and mineralization, which is abolished by rh Noggin, a BMP antagonist [97].

Cultured dental follicle precursor cells were differentiated into osteogenic cells in the presence of ascorbic acid, dexamethasone, and inorganic phosphate and harvested after four weeks [105]. 2-DE/LC–MS proteomics revealed 115 differentially expressed proteins during osteogenic differentiation. The most upregulated proteins were beta-actin, glutamine synthetase, lysosomal proteinase cathepsin B proteins, plastin 3 T-isoform, superoxide dismutases, and transgelin. Bioinformatics analysis showed that these proteins are mainly involved in catabolism, cell motility, and biological quality. The most downregulated proteins were cofilin-1, destrin, dihydrolipoamide dehydrogenase, pro-alpha 1 collagen, and prolyl 4-hydrolase, which are associated with collagen biosynthesis, cell cycle progression, and protein metabolism.

Dental pulp isolated from murine molars has an abundance of cells expressing CD90/CD45$^-$, CD117/CD45$^-$ and Sca-1/CD45$^-$. These cells can readily differentiate into osteogenic cells but fail to differentiate into chondrocytes or adipocytes [106]. There are, however, other populations of pluripotent dental pulp stem cells that are capable of differentiating into adult tissues from all three embryonic layers; i.e., endothelial cells, neurons and hepatocyte-like cells [107, 108]. These cells are characterised as follows: SSEA-4$^+$, OCT4$^+$, NANOG$^+$, SOX2$^+$, LIN28$^+$, c-Myc$^+$, CD13$^+$, CD105$^+$, CD34$^-$, CD45$^-$, CD90 low, CD29$^+$, CD73 low, STRO-1 low, and CD146$^-$. VEGF promotes proliferation when applied in undifferentiating conditions but enhances osteogenic differentiation in osteogenic media conditions. Furthermore, 5-Aza has been shown to turn dental pulp cells into skeleton muscle cells, similar to BMSCs, which was discussed in Sect. 5.3.

Proteomics was used to identify changes to the golgi apparatus during osteogenic differentiation of dental pulp–derived MSCs [109]. The analysis showed that 39.3 % of proteins are upregulated and 16 % of proteins are downregulated; 4 % of proteins are newly synthesised. A 2D-gel proteomics study was used to investigate the protein profile of dental pulp cell undergoing differentiation into odontoblast-like cells [110]. This study showed that 23 proteins were significantly altered and bioinformatic analysis revealed that some of these proteins regulate cytoskeleton, matrix synthesis, and cellular metabolism; others were nuclear proteins and cell membrane-bound molecules that may be involved in signaling pathways. Changes of four proteins—heteronuclear ribonuclear protein C, annexin VI, collagen type VI, and matriline-2—were verified by Western blotting and real-time PCR. The data provide a foundation for further studies to explore the process

taking place during the differentiation of dental pulp cells into odontoblast-like cells.

Periodontal ligament cells have been shown to be able to differentiate into osteocytes for use in regenerative medicine. Growth factors such as IGF-1, PDGF, and BMP-2 can increase the capacity of these cells to form new bones [111]. Using DIGE proteomics, we characterized the protein profile during osteogenic differentiation of the MSCs from periodontal ligaments and identified 29 altered proteins. Of these, 12 proteins were upregulated and 17 proteins were downregulated [112]. The majority of these proteins are associated with the regulation of cytoskeleton. Decrease expression was found in caldesmon, tropomyosin, and heterogeneous nuclear ribonucleoprotein C, whereas increased expression was found in the calcium-binding protein annexin A4. These findings add further to our understanding of the mechanisms governing the osteogenic differentiation of MSCs from periodontal ligaments.

7.3 Pancreatic Islets

Beta-cells play an important role in sugar metabolism, which can lead to diabetes. MSCs isolated from pancreatic islets can be used for beta-cell differentiation [113, 114]. A number of studies have shown that MSCs from different origins share a similar profile. However, islets formed from islet-derived MSCs compared to islets formed from bone marrow stromal cells are different with respect to PDX1 (pancreatic duodenal homeobox gene-1), insulin, C peptide, and Glut-2, which are not expressed by BMSC islet cells; these cells only expressed Glut-2 and insulin. PDX1 and NGN3 play a key role in beta-cell lineage development from telomerase immortalised MSCs [115, 116].

7.4 Umbilical Cord and Placenta

Umbilical cord (UC) and umbilical cord blood (UCB) are both sources of MSCs, although MSCs are far easier to obtain from UC than UCB. MSCs from both sources have the same potential for osteogenic, adipogenic, and chondrogenic differentiation [117–119]. Proteomics was used to study the cell migration ability of MSCs sourced from bone marrow, UC, and UCB. MSCs from human bone marrow, umbilical cord, and placenta were shown to be different with BMSC and were 5.9 fold higher than UC-MSC [61]. The reasons were elucidated by proteomic study, which identified 6 proteins expressed differently in these cells. Both BM- and P-MSC produced little PAI-1 and overexpression of PAI-1 in these cells decreased the migration abilities. UC-MSCs produced much higher PAI-1 and silencing of PAI-1 increased their capability to migrate.

8 Future Directions

Proteomics has proved to be a valuable resource in the study of MSCs and promises to produce data that will help elucidating the biology of these versatile cells. The vast majority of proteomics have adopted the tried and tested methods, with most relying on 2D-gel and MALT-TOF. New and more powerful technologies are consistently being developed and released into the market; these new systems will undoubtedly produce exciting new data that will help researchers in the quest to unlock the full potential of MSCs. Technologies such as DIGE and iTraq are already proving their usefulness by being able to assay protein expression in several samples simultaneously.

The study of protein modification still remains outside the purview of most proteomics techniques. For example, the phosphorylation status of proteins reflects intracellular signaling events, thus representing functional status. However, these molecules are expressed in very low amounts and are therefore below the detectable range of ordinary proteomics. Phosphylation proteomics can be used to reveal proteins changed in phosphorylation status [120]. These data could be highly valuable for the elucidation of mechanisms for MSCs differentiation and migration.

The utility of proteomics greatly increases when combined with other techniques to elucidate the role of differentially expressed proteins. The biological function of proteins flagged by initial proteomics screens can only be understood when viewed from a number of different angles. In vitro and in vivo knockout and overexpression studies are experimental approaches that have the greatest potential to unlock the function of such proteins.

9 Conclusions

Proteomics has emerged as one of the most powerful tool available to scientists for high-throughput protein screening. It has been particularly useful in the study of MSCs in the quest to solve major issues in this field of research, such as the identification of MSC-specific markers, the regulation of the differentiation potential of MSCs into various terminal cell types, and the mechanisms underlying the effects of various growth factors to stimulate MSCs. The suite of proteomics techniques has provided a vast amount of valuable information. However, much work still remains before we fully understand the biology and signaling pathways that govern the behaviors of MSCs. The search for this knowledge is not merely an academic exercise but has great clinical relevance as the science of tissue engineering comes closer to developing real clinical solutions for patients' needs.

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Does the Adult Stroma Contain Stem Cells?

Richard Schäfer

Abstract It is well accepted that adult mesenchymal stromal cells (MSCs) comprise subpopulations of cells sharing common phenotypical and functional properties. However, there is emerging evidence that MSC subpopulations may also feature distinct characteristics. This chapter focuses on MSC subpopulations reflecting their possible stem cell properties relative to defined pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). This attempt at an ontogenetic reflection on MSCs can be useful for both basic and translational research in the field.

Keywords Bone marrow · Differentiation · Mesenchymal stromal cells · Pluripotent · Stem cells

Contents

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1 Stem Cells and Multipotent Cells

In order to discuss stem cell properties of MSCs in relation to stem cells it is useful to summarize the characteristics that define the latter.

Stem cells are self-renewing cells that retain their stem-cell properties in the daughter cells upon cell division [1]. Self-renewal, regulated by TGFβ, JAK/STAT, Wnt, and Notch signaling pathways and factors such as leukemia inhibitory factor (LIF) and bone morphogenetic proteins (BMPs), is associated with the ability to maintain clonal cell growth [2, 3]. As clonal cell growth comes as colony formation in vitro, assays were developed to assess the content of stem cells within a cell preparation by quantifying the colony-forming cell clones [3]. However, clonal growth per se is not sufficient to assign cells a "stem cell character" as the ability to form colonies in vitro is shared by various cell types that can be isolated for example from bone marrow (BM) [4].

Besides self-renewal, stem cells are characterized by their ability to differentiate into more specialized (lineage committed) cell types. Totipotent stem cells, being present only up to the 8-cell stage in the early embryo, can generate a complete organism. Pluripotent stem cells (PSCs) like embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) can give rise to cells and tissues of all three germ layers and to cells supporting embryonic development [5]. Stem cell pluripotency can be assessed by analyzing the in vivo teratoma formation capacity of these cells after transplantation into an immunodeficient host organism [6, 7].

Multipotent (stem) cells, currently still poorly defined, exhibit a limited differentiation capacity that is restricted to a small variety of cell types. Moreover, they show a lower self-renewing capacity and a limited lifespan compared to PSCs [8, 9]. Multipotent cells are considered to already represent a lineage- or at least germ layer-committed cell pool that provides cellular homeostasis in adult organs. Therefore, it is debatable whether or not multipotent cells can be regarded as "stem cells" or if the label "stem cells" should be reserved for cells that meet the criteria of PSCs.

As outlined above, stem cells are functionally and most importantly characterized by their self-renewal and pluripotent differentiation capacity. In the past decade, significant progress was made to elucidate the molecular signature of PSCs. Hereby, several transcription factors have been identified that have been shown to maintain the pluripotent state of PSCs mainly by inhibiting differentiation. Consequently, these factors were depicted as "pluripotency associated genes": Oct4, Sox2, Nanog, Rex1, and SALL4 [10–12]. Some of these transcription factors are functionally arranged in circuits: The Oct4-Sox2 circuit and the Nanog-SALL4 circuit [13, 14]. It has to be pointed out that among the four Oct4 isoforms it is only Oct4A that has been shown to maintain pluripotency in ESCs (see below) [15, 16]. Additionally, surface markers have been described that were found to be useful as surface "stem cell markers" because these antigens were expressed on PSCs but not on their differentiated progeny: SSEA-4, TRA-1-60, and TRA-1-81 [17]. Recently, the identification of a PSC subset, defined by the

expression of SSEA-5, which is highly capable of teratoma formation proved for the first time the heterogeneity of cells within PSC colonies [7]. Whether or not the reported variations of differentiation propensity between PSC lines [18] might be a result of the already given heterogeneity within PSC colonies remains to be elucidated.

Yamanaka and Thomson introduced re-programming generating iPSCs from differentiated adult somatic cells by transducing with Oct4, Sox2, Klf4, and c-Myc (Yamanaka) or Oct4, Sox2, Nanog, and Lin28 (Thomson) [19, 20]. Meanwhile, a variety of integrative and nonintegrative re-programming technologies has been developed [21].

2 The Story of Mesenchymal "Stem" Cells

As our current understanding of the adult stroma is mainly based on the BM, the following refers to BM-derived stromal cells.

In the early 1970s, Alexander Friedenstein described colony-forming units of fibroblastoid (CFU-F) cells that could be isolated from the BM stroma supporting hematopoiesis and osteogenesis [22–25]. Numerous reports on mesodermal in vitro differentiation capacity of stromal cell preparations including adipogenic, osteogenic, chondrogenic [26], and myogenic [27, 28] lineages led to the introduction of the term "mesenchymal stem cells" [29]. The contribution of MSCs to the "mesengenic process"—that is the continuous generation of cells that ensure the supply of mesodermal tissue in adult organisms—supported the concept of MSCs as highly plastic cells that are capable of generating a wide variety of cell types and tissues [30].

The encouraging results of the in vitro differentiation experiments, the regenerative potential of MSCs after in vivo transplantation into various defect and disease models [31, 32], and simple isolation and expansion technology shaped the first two euphoric decades of MSC research promising a bright future for MSC-based regenerative medicine.

However, emerging reports heralded a more sobering phase in the field:

- *Lack of proof of functional non-mesodermal differentiation or myogenic differentiation*

In vitro and in vivo studies reported on "transdifferentiation" of MSCs into cells featuring phenotypical properties of neuroectodermal cells [33, 34]. However, there is lack of evidence on neuron-typical properties of "transdifferentiated" MSCs such as formation of functional filaments, synaptic connectivity, or adequate electrophysiological properties [32]. Despite reports on differentiation of MSCs into cardiomyocytes [35–37], the differentiation of functional myogenic cells from MSCs has been questioned: MSCs cultured with "differentiation media" expressed mRNA and proteins associated with the cardiomyogenic lineage (e.g., cardiac

troponin I, atrial natriuretic protein, cardiac alpha sarcomeric actin, slow muscle myosin, and myosin light chain), however, MSCs cultured with standard media did so as well and no functional cardiomyocytes (beating cells, functional contractile elements, specific electrophysiological properties) could be generated from MSCs [38, 39]. The ability of myocytes to fuse with other cell types [40] raised the question whether the "myogenic differentiation" of MSCs in vivo might rather be the result of fusion with resident myocytes than real myogenic differentiation of MSCs [41–43].

- *Heterogeneity of stromal cell preparations in vitro and within the stroma in vivo*

Although cells within MSC preparations in vitro share some phenotypical properties (fibroblastoid morphology, expression of CD73, CD90, and CD105, and absence of hematopoietic markers [26]) it became evident that MSC preparations in vitro are composed of subpopulations that are still poorly defined, and only a minority of clonally expanded MSCs show a broader (adipogenic, osteogenic, and chondrogenic) mesodermal differentiation potential [4, 44–46]. To date, surprisingly few studies provide evidence that the heterogenic situation in vitro may be reflected by heterogeneity within the stroma, so far identifying the perivascular region as the main location of MSCs in vivo [47–51].

- *In vitro artifacts upon extended culture*

José Diaz-Romero et al. [52] showed that chondrocytes can change their phenotype and gene expression profile when cultured as a monolayer in vitro. On MSCs, antigens such as CD29 and CD90 are highly expressed during in vitro culture, whereas CD45 and HLA class 2 antigens are not detectable [46]. However, directly after isolation, MSC preparations contain cells expressing CD45 [53, 54] but not CD29 [54] on their surface. Moreover, the expression of HLA class 2 antigens is regulated by interferon gamma [55, 56], and the expression of CD90 can decrease on transformed MSCs [57]. Currently, it is unclear if the "loss" and "gain" of antigen expression in stromal cell preparations is the result of selection processes or the result of regulation processes in single MSCs during culturing. Certain MSC subsets (e.g., multipotent adult progenitor cells [MAPCs, see below]) require sophisticated culture conditions [58]. Therefore, they are not routinely detected in stromal cell preparations under standard culture conditions (e.g., alpha MEM + 10 % fetal bovine serum or human serum; 21 % O_2) and occasionally these elements are regarded as "culture artifacts" without an in vivo correlate [59]. However, "standard culture conditions" might not reflect the in vivo situation [e.g., the oxygen partial pressure in the BM (55 mmHg) is much lower than in air (159 mmHg) [60], therefore it is not unlikely that rare MSC subpopulations such as MAPCs are not captured by "standard culture conditions" but might exist in vivo.

- *Different results with MSCs from different sources*

MSCs can be isolated from nearly all stromal tissues in the adult organism [61]. However, MSCs derived from the two main sources, that is BM and adipose tissue

(AT), differ in, transcriptome, proteome, and phenotype [59]. Compared to BM-derived stromal cells, AT-derived stromal cell preparations contain cells expressing the CD34 antigen and fewer cells expressing the adhesion molecule VCAM-1 [59, 62]. Interestingly, no clear evidence exists on a distinct differentiation propensity of MSCs derived from BM or AT [59].

- *Different results with MSCs from different species*

MSCs can exert xeno- or alloreactivity [63–65]. Therefore, syngeneic animal models are valuable tools especially for transplantation studies. However, the antigen expression profile of BM-derived human, pig, rat, and mouse MSCs is not identical [46, 66, 67]. Murine MSCs lack the expression of CD90, whereas human, pig, and rat MSCs highly express CD90. Moreover, strain-to-strain variations of antigen expression are reported in mouse MSCs (e.g., Sca-1) and rat MSCs (e.g., CD44). These results suggest that the stroma of different species and animal strains might be composed of different cell types hampering the comparability of MSCs between species.

It has to be pointed out that the aforementioned points have been controversially discussed in the field and controversial data is still being produced.

3 MSC Subpopulations

3.1 Phenotypical Variety in Stromal Cell Preparations

BM stromal cell preparations are commonly generated by culturing the adherent cells from the mononuclear cell fraction of the BM using density gradient technology [68]. The surface antigen pattern defining MSCs as proposed by the International Society of Cellular Therapy (ISCT) includes the positive expression of CD73, CD90, and CD105 and the absence of hematopoietic markers such as CD34, CD45, CD14 or CD11b, CD79a or CD19 [26]. As CD73, CD90, and CD105 are usually expressed on all the cells (>95 %) within an MSC preparation in vitro, flow cytometry and immunocytochemistry analysis reveal that other antigens such as GD2, CD173, CD271, or tissue-nonspecific alkaline phosphatase (TNAP) can be detected on fractions of cells within a stromal cell preparation [46, 69]. Moreover, by microscopical or flow cytometry analysis of stromal cell preparations at an early stage after starting the in vitro culture (passage 0 and 1) adherent cells with different morphology (slim, spindle shaped vs. broad, round; FS^{lo}/SS^{lo} vs. FS^{lo}/SS^{hi} vs. FS^{hi}/SS^{lo} vs. FS^{hi}/SS^{hi}) can be identified [70]. These observations point towards the presence of distinct MSC subpopulations within stromal cell preparations in vitro. As outlined above, very few studies investigated the distribution of MSCs within the stroma in vivo assigning the perivascular region as the main location of MSCs [47–51]. However, together with Massimo Dominici's group in Modena, Italy we identified mesenchymal stromal cell entities

by different morphology and microanatomical localization in relationship to a distinct pattern of antigen expression in the human BM in vivo. This study showed that morphologically different MSCs are not exclusively located in a perivascular position but also in the endosteum, in the adipose tissue, and in the medullary cavity of the BM. Moreover, rare elements could be located in the medullary cavity of the BM expressing Oct4, Nanog, and SSEA-4. To date it is unclear if and how the different stromal cell entities located in the human BM can be expanded and analyzed in vitro. Another study representing a further step towards functional characterization of MSC subpopulations reported on the analysis of mRNA expression of 96 genes in $CD45^{-/low}CD271^{bright}$ MSCs directly after sorting from fresh BM. Compared to cultured MSCs and hematopoietic cells, $CD45^{-/low}CD271^{bright}$ MSCs showed an increased expression of Wnt-related genes (including Nanog and Oct4) and genes involved in adipogenesis (FABP4, PPARγ) and osteogenesis (osterix, osteonectin, and osteopontin) [53].

In addition to MSC subpopulations defined by phenotype, the following stromal cell entities have been described and depicted with distinct denominations:

3.2 MAPCs

Multipotent adult progenitor cells (MAPCs) were isolated from the total cell fraction obtained from flushed bone fragments (no density gradient technology used) and cultured using sophisticated media formulations [58, 71]. MAPCs isolated from mouse and rat BM could be long-term cultured without showing evidence of senescence (stable telomere length) in vitro. They could be differentiated into cells showing not only another mesodermal (endothelial), but also the neuroectodermal and endodermal phenotype in vitro. MAPCs transferred into blastocysts gave rise to a great variety of differentiated cells and when transplanted into NOD/SCID mice, MAPCs engrafted and were shown to differentiate into cells of mesodermal (hematopoietic cells) and endodermal (liver, gut, and lung epithelium) but not ectodermal lineage in vivo. Compared to mouse ESCs, mouse MAPCs expressed a similar amount of *Rex-1 mRNA* but much less *Oct4 mRNA* [71]. Human MAPCs were shown to differentiate into a great variety of mesodermal cell types, expressed *Oct4 mRNA* and protein at variable levels, but no *Nanog mRNA* or *SOX2 mRNA* [58, 72]. Moreover, MAPCs transplanted into sublethally irradiated NOD-SCID mice engrafted and showed long-term multilineage hematopoietic reconstitution [72]. Although no in vivo correlate for MAPCs has been described so far, MAPCs isolated from rodent BM might appear as a rare stem cell entity within the adult stroma; however, human MAPCs might merely be regarded as a multipotent MSC subpopulation due to the restricted differentiation potential.

3.3 MIAMI Cells

D'Ippolito et al. isolated cells from whole BM (no density gradient technology used) that was obtained from vertebral bodies of deceased patients [73]. Similar to MAPCs, these cells required optimized culture conditions in vitro for example extracellular matrix (fibronectin), low (= physiological?) oxygen tension (3 % O_2), growth factors (basic-fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, β-nerve growth factor, neurotrophin-3, brain-derived neurotrophic factor, transforming growth factor-β3), and other proteins (activin-A, exendin-4). Depicted as marrow-isolated adult multilineage inducible (MIAMI) cells, they showed a prolonged proliferation capacity and positive expression of *human telomerase reverse transcriptase mRNA* pointed towards lack of senescence. MIAMI cells expressing *Oct4* and *Rex1 mRNA* could be differentiated in vitro into cells of osteogenic, chondrogenic, adipogenic, neural, and endodermal (pancreatic β-cell like) phenotype. Importantly, the targets that were regarded to be indicative for lineage-specific differentiation (neuronal nuclear protein, neurofilament 160 (neural phenotype), insulin, glucagon [pancreatic β-cell-like phenotype]) could be detected in differentiated but not in undifferentiated MIAMI cells.

3.4 VSEL Stem Cells

Very small (diameter: 3–6 μm) Sca-1$^+$lin$^-$CD45$^-$ cells can be sorted from murine BM after red cell lysis and from human cord blood [74, 75]. Depicted as very small embryonic-like (VSEL) stem cells they express *Oct4*, *Nanog*, *Rex-1*, and *SSEA-1 mRNA* as well as Oct4, Nanog, and SSEA-1 protein/antigen. BM-derived VSEL stem cells were differentiated in vitro into cells of mesodermal, endodermal, and ectodermal phenotypes. Compared to undifferentiated VSEL stem cells, VSEL stem cell derivatives showed an increased expression of *troponin I*, *cardiac myosin binding protein-c 3*, *sarcomeric actinin 2* and *3 mRNA* ("cardiomyocyte-like"), *nestin*, *olig* 1 and *2 mRNA* ("neuron-like, oligodendrocyte-like"), and *glucagon*, *insulin*, and *amylase 2 mRNA* ("pancreas-like"). VSEL stem cells transplanted into lethally irradiated syngeneic animals did not reconstitute hematopoiesis or contribute to hematopoiesis and were not able to form teratomas in vivo highlighting important functional differences to PSCs [74, 76]. Moreover, VSEL stem cells did not proliferate in vitro without being co-cultured on BM-derived stromal cells as a feeder layer. Shin et al. [76] analyzed the DNA methylation pattern of VSEL stem cells. Addressing the Oct4 pseudogene problem (see below), they analyzed the DNA methylation status of the Oct4 promoter. In VSEL stem cells the Oct4 promoter was hypomethylated whereas in hematopoietic stem cells and unsorted stromal cell preparations the Oct4 promoter was hypermethylated. Oct4 promoter hypomethylation provides important supportive evidence for the actual transcription of the Oct4 gene in VSEL stem cells. To address the "proliferative quiescence" of VSEL stem

cells that remains in contrast to the highly proliferative capacity of PSCs, they analyzed the methylation pattern of differently methylated regions (DMRs) known to regulate the expression of imprinted genes that are important in development. In VSEL stem cells paternally methylated DMRs were shown to be "erased" but maternally methylated DMRs were found to be hypermethylated. Referring to the fact that paternally expressed imprinted genes promote cell proliferation, but maternally expressed imprinted genes suppress cell proliferation the authors of this study concluded that the specific DMR methylation pattern of VSEL stem cells might be responsible for their impaired growth in vitro [76].

4 Do We Use the Right Markers to Identify Stem Cells in Stromal Cell Preparations?

As outlined above, Oct4 and Nanog are highly expressed by PSCs and these transcription factors are involved in the maintenance of pluripotency. Moreover, a recent study by Tsai et al. showed that Oct4 and Nanog regulate self-renewal in hBM-MSCs [77]. Therefore, in a number of studies the possible "stemness" of stromal cells was assessed by simply analyzing the expression of these targets on the mRNA or protein level. As outlined above, among the four Oct4 isoforms it is only Oct4A that has been shown to maintain pluripotency in ESCs [15, 16]. Amongst the great number of studies reporting on Oct4 expression in MSCs only a few studies actually analyzed Oct4A expression for example in hBM-MSCs [78] or hMIAMI cells [79]. Therefore, it is important to use a methodology (primers, antibodies) that can either distinguish between the three Oct4 isoforms or at least reliably detect the Oct4A isoform. Moreover, it is known that Oct4 and Nanog pseudogenes are expressed in the cytoplasma but not in the nucleus where the pluripotency-associated transcription factors are located [77].

5 Outlook: Proposed Experiments to Investigate the Presence of Stem Cells in MSC Preparations In Vitro and/or in the Stroma In Vivo

The adult stroma is composed of multiple cell entities sharing some phenotypical and functional properties. The vast majority of these cells can most likely not be classified as stem cells but multipotent cells. However, despite issues of lab-to-lab variability and challenges of reproducibility, there is emerging evidence that the adult stroma might harbor rare elements that feature stem cell properties.

To address this question, future studies are encouraged to set the same rigorous standards for MSCs that are applied to PSCs.

- Identify/isolate MSC subpopulations by phenotypical patterns (marker combination) that were identified in vivo (murine markers might not be applicable in the human system).
- Ideally start analysis from single cells and continue at the clonal level.
- Both MPACs and MIAMI cells require initial co-culturing with other (not yet specified) BM cells as whole BM was plated without the use of density gradient technology. Future experiments designed to identify/isolate MSC subpopulations would have to address the possible dependence of MSC subpopulations on other stromal cell entities.
- Develop culture conditions allowing the growth of MSC subpopulations without changing their properties (real in vitro artifact).
- Confirm that "pluripotency markers" are detected in the same compartment as they are located in PSCs (transcription factors [Oct4A, Nanog] in the nucleus and not in the cytoplasma).
- Apply the appropriate tools to assess functional stem cell properties:

  - Blastocyst transfer followed by analysis of in vivo chimerism and assessment of all-lineage differentiation.
  - Teratoma formation in vivo.
  - Re-constitution or at least contribution to hematopoiesis in vivo.
  - Assessment of functional differentiation capacity (e.g. electrophysiology, detection of contractile elements). The detection of "lineage-specific proteins" might not be sufficient as they can be expressed also by undifferentiated MSCs [38].
  - Rule out cell fusion events.

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Index