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Mesenchymal Stromal Cells and Fibroblasts

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Summary

Adult mesenchymal stromal cells (MSC), also referred to as mesenchymal stem cells, were detected almost half a century ago in bone marrow and have been studied intensively in the last decade. Different aspects of MSC biology were explored and published. Studies pointed to their localization in different organs during development and in adulthood and described their characteristics in experimental or clinical investigations. Despite intensive research in the field and in sharp contrast to hematopoietic stem cells (HSC), it has become more and more clear that MSC lack a unique cell surface marker. MSC not only share cell surface markers with other types of cells, they also share many features with pericytes and fibroblasts, including their capability to differentiate into, for instance, osteoblasts or adipocytes. In this review we therefore screen the current literature to disclose differences between MSC and fibroblasts and also report on common qualities.

Introduction

Almost 140 years ago, in 1876, changes in the appearance of bone marrow were associated with anemia suggesting that some blood cells might arise from this source [1]. In 1867 the same author, Prof. Cohnheim, had already reported that dye-labelled fibroblast-like cells migrated to sites of injury and inflammation during would healing processes [2]. He concluded that at least some of the invading cells involved in tissue regeneration might be derived from blood or bone marrow. Basically a century later, in 1966, Friedenstein described an osteogenic population of cells isolated originally from bone marrow [3] and several studies from Friedenstein's laboratory portrayed the cells, we call MSC today, in more detail [4-6]. Back then of course an in depth characterization of the expression of cell surface markers and discrimination between several distinct lines or types of MSC was impossible for a simple reason. The generation of monoclonal and highly specific antibodies to defined cell surface structures and proteins was developed in the seventies and published in 1975 [7]. In addition, in the first decade of research with this new tool, most monoclonal antibodies were generated to cells involved in immune responses or related to malfunction of the immune system. Therefore knowledge on hematopoietic stem cells (HSC), the expression of cell surface antigens on HSC or the many blood borne cells in association with differentiation pathways led the field of cellular research since. Even activation or inactivation of immune cells during inflammation and infection, in autoimmunity or in hematopoietic diseases can often be explored with monoclonal antibodies nowadays. This is different for MSC: Antibodies to isolate, characterize and possibly even separate MSC from their progeny, including fibroblasts, osteoblasts, chondrocytes, adipocytes and others or reagents to distinguish functional subsets within MSC preparations are not readily available.

But in recent years more and more studies generated a solid foundation that differentiation-competent MSC reside not only in bone marrow, but also in other tissues and MSC were isolated from different origins [8-10]. In 1999 the description of human bone marrow-derived MSC (bmMSC) expressing the antigens SH2 (CD105) and SH3 (CD73), but lack antigens characteristic for monocytes (CD14), HSC (CD34, CD133) or endothelial cells (CD31, CD34) and leukocytes (CD45) initiated an avalanche of studies and publications on this novel topic [11,12]. The differentiation capacity of MSC to generate osteoblasts, chondrocytes and adipocytes *in vivo* or *in vitro* [12] was corroborated by many studies. Some laboratories claimed an even wider differentiation capacity. Among others, generation of muscle cells [13], neuronal cells [14] and endothelial cells was described [15]. But the overall efficacy of generating differentiated cells from MSC was and is very variable [16]. This may be due to several reasons:

i. The protocols for *in vitro* differentiation of MSC are suboptimal.

ii. During *in vitro* expansion MSC change or lose the original differentiation capacity [17].

iii. Bulk MSC contains distinct subsets of predetermined cells that preferably differentiate towards one cell line but not to any other type of cell [18].

iv. Efficient differentiation requires a complex blend of biological, chemical and physical stimuli, including:

a. Soluble signals (hormones, growth factors, low molecular weight compounds, O_2 and NO-content, etc.).

b. Engagement of the cellular niche (extracellular matrix composition [19], integrin anchorage of cells, matrix nano-patterns, cell-cell interactions, etc.) [20].

c. Elasticity of the environment [21].

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Thus, there is experimental evidence that all of the above contribute to MSC differentiation [18,22-24] (Figure 1). However, there is even one more important integral element to take into account: the source of MSC [25]. Cells sharing many features with MSC can be isolated from virtually all tissues even in adult donors [8]. Despite the similarities of the MSC derived from different origins, it has been noted that the differentiation capacity of MSC varies considerably among cells isolated from different sources [25,26]. To shed some light on the biology of MSC, consensus conferences coined some criteria to better define MSC from bone marrow (bmMSC) [27,28] or placenta (pMSC) [29,30]. But an in-depth methodical examination to define MSC from adipose (aMSC) [31] or other tissues was not presented [8,25,32-34]. Although the differences between MSC from different sources constitute an interesting field for research and reflection, in this review we discuss expression of cell surface markers to discriminate MSC from other cells and address the problems and recent studies of multi-lineage differentiation of both MSC and dermal fibroblasts (DF).

Differences in gene expression

To discriminate human DF from human bmMSC, gene expression analyses have been performed [35]. In these studies all known transcripts of the whole human genome were investigated by a microarray technique allowing an in-depth screening of the gene expression of a given sample. The authors report that 64 genes defined the molecular signature of MSC in comparison to fibroblasts. All gender-related genes were eliminated and a minimum of a 10-fold difference in steady-state transcript levels between DF and bmMSC was applied [35]. Statistical evaluation of replications of sets of probes indicated that the reproducibility was satisfactory (i.e. p 0.065). Interestingly, many of the transcripts that were expressed differently

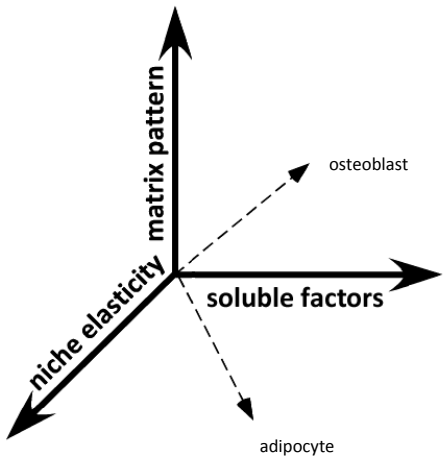

Figure 1: Stimuli inducing and modulating the differentiation of mesenchymal stromal cells. MSC reside in niches and their proliferation and differentiation is regulated by (x) soluble factors, including cytokines, hormones, growth factors, and low molecular weight components, by (y) the extracellular matrix, its composition and cell-cell interactions, and by (z) the elasticity of the niche. The osteogenic differentiation is for instance facilitated in a less elastic, rigid environment, in combination with collagen signalling, and appropriate cytokines (upper dotted pathway). In a soft, more elastic environment, soluble factors and matrix signals will rather generate adipocytes (lower dotted pathway) [21]. However, it seems that the general direction of differentiation is set by the soluble factors, and that the extracellular matrix and elasticity of the niche modulate the efficacy of differentiation.

in DF vs. bmMSC coded for cell membrane proteins. Among them the plexin domain-containing protein 1 (PLXDC1, expression ratio MSC > DH, 37-fold), also referred to as tumor endothelial marker 7 (TEM7). PLXDC1 plays an important role in generation of neovasculature and it molds capillary morphogenesis. It is associated with high risk of metastases and poor prognosis in patients with osteosarcoma. Another membrane protein, N-acetylgalactosaminyl-transferase 3 (GALNT3, expression ratio MSC > DH, 30-fold), is an enzyme involved in oligosaccharide biosynthesis. Mutations of GALNT3 have been associated with pathology in mineralization of bone, high risk of bone fracture and even mineral deposition in soft tissues (calcinosis). Another interesting factor reported to be expressed differently is vascular cell adhesion molecule 1 (VCAM1, expression ratio MSC > DF, 20-fold) [35]. VCAM1 was given the cluster of differentiation (CD) CD106 and in another study a 6-fold higher mRNA expression was observed in MSC in early passages [36]. CD106 is expressed by endothelial cells in both, large and small blood vessels. It mediates the interaction between the vessel wall and different cells and is the principle ligand to integrin α4β1 or α4β7. Expression of CD106 was reported on MSC from bone marrow [18], placenta [37], but not on MSC from adipose tissue [38]. CD106 is normally not expressed on DF [39]. Therefore *prima vista* expression of CD106 seems to discriminate between MSC and fibroblasts. We will inspect this hypothesis in the next chapter, when we elaborate differences between MSC and DF with regard to expression of cell surface proteins.

In addition, fibroblast growth factor receptor 2 (FGFR2, CD332) and vascular endothelial growth factor A (VEGFA) were both expressed in MSC approximately 10-fold higher compared to DF [35]. FGFR2 was associated with regulation of osteogenesis. Therefore an elevated expression of FGFR2 on bmMSC is not surprising. Regulation of VEGFA is activated in MSC by low oxygen tension [40] and hypoxia was shown to facilitate its expression in fibroblasts as well [41]. Hence this factor is also not a prime candidate to discriminate DF from MSC.

Expression of transcripts encoding the alpha-11 chain of integrin (7-fold) and integrin-like growth factor-2 were also elevated (5-fold) in early passage MSC compared to DF. In contrast, expression of interstitial collagenase (MMP1) and stromelysin (MMP3) were elevated 8-and 6-fold in DF compared to MSC, respectively [36].

Regrettably, the studies investigating the transcriptome in MSC vs. DF [35,36,42] utilized cells from commercial sources. So the term "early passage" has to be put in perspective to the history of the individual batch of MSC. Thus, the number of cell divisions prior to the actual experiment may vary. Moreover, expansion media and other variables influence the outcome of the studies as well. Therefore determination of differences between MSC and DF should possibly be standardized by defining variances and consistencies between MSC and DF *ex vivo*. Here of course, monoclonal antibodies enter the stage. But isolating MSC and especially DF from tissue puts stress on the cell and generally involves proteolytic enzymes at some stage. Note that even mild proteolysis may harm structures on cell surfaces. Thus, this strategy of *ex vivo* characterization of MSC versus DF has its disadvantages as well.

Surface markers of MSC and fibroblasts

As pointed out above, investigation of the whole transcriptome on human MSC and DF to determine differences between these cell types is a very interesting scientific challenge, at least scholarly [35]. For practical purposes and especially for tissue engineering in a pre-clinical or clinical setting, a clear-cut discrimination or even separation of MSC

from fibroblasts, osteoblasts, adipocytes by a cell surface determinant would improve our proposition.

According to consensus conferences [27,28,30], bmMSC express CD73, CD90, CD105 and lack expression of antigens found on monocytes (CD11b, CD14), on HSC (CD34, CD133), endothelial cells (CD31, CD133) or on leukocytes (CD45). The bmMSC also lack expression of the major histocompatibility antigens HLA class II. However, this pattern of expression of cell surface antigens ($CD73^+$, $CD90^+$, $CD105^+$, $CD11b^-$, $CD14^-$, $CD31^-$, $CD34^-$, $CD45^-$, HLAclassII$^-$) was observed with DF too. But there seems to be at last distinct patterns of antigen expression discriminating e.g. bmMSC *ex vivo* ($CD271^+$, $TNAP^+$, SSEA-4^-, TRA-1-81^-) [22-24] from other MSC or from DF. For instance, adipose tissue-derived MSC express *ex vivo* and in early passages of *in vitro* cultures CD34 but lack expression of CD271 [43], whereas MSC from the amnion membrane express SSEA-4 and TRA-1-81, but not CD271 [44, 45]. Therefore expression of CD271, TNAP, SSEA-4, TRA-1-81, or CD34 can be utilized to at least discriminate DF from some MSC ex vivo.

As outlined in the previous paragraph, expression of CD106, alias VCAM1, encoding mRNA was significantly different in MSC compared to DF [35]. But determination of CD106 by flow cytometry reports that only 2% of bmMSC and 10% of pMSC express CD106 on the cellular surface [46]. On normal DF, CD106 is not detected by flow cytometry. But it can be activated by different stimuli [47]. Furthermore, on MSC expression of CD106 is fading *in vitro* [36]. So on MSC its expression depends on the culture conditions. Thus despite significant differences in mRNA expression rates of CD106 between MSC and DF [36], expression of the CD106 protein varies on both MSC and DF depending on culture conditions. In addition, DF was shown to be differentiation competent [48]. Therefore expression of CD106 is not a robust marker to discriminate MSC from fibroblasts.

Recently, fibroblast-activation protein alpha (FAPα) was reported to be expressed on bmMSC but not on other bone marrow-derived cells [49]. But spontaneous expression of FAPα was rather high in normal human DF, although the mean of fluorescence intensity was further increased significantly in keloid DF from scar tissue [50]. Therefore, as stated for CD106, FAPα seems not to be a feasible marker for discriminating MSC from fibroblasts.

Moreover, expression of CD146 was observed on bmMSC, but not on DF, osteoblasts, periosteal cells, nor on cells derived from fibrotic bone marrow [51]. This suggested that CD146 might serve as marker to discriminate MSC form DF. We corroborated that bmMSC express CD146 and CD146 was observed on bmMSC at higher levels compared to pMSC [26]. But our preliminary studies indicate that DF expresses CD146 at levels close to pMSC, at least when expanded in the same medium as the MSC (Ulrich, doctoral thesis, Eberhard-Karls-University, Tübingen, Germany (in preparation)). A current compendium on expression of MSC cell surface markers concluded that there is great discrepancy in the literature [52]. For science these inconsistencies are a challenge tempting our curiosity and therefore academic sport. For colleagues involved in tissue engineering and production of MSC for therapeutic regimen, lack of clear MSC markers become a difficult problem.

Differences in expression of small RNA species

In addition to investigating differences in steady state amounts of mRNA between MSC and DF [35,36], differences were also explored in the pools of microRNA's (miRNA) [35]. In contrast to the mRNA, which is a complex population of messengers encoding all proteins required for proper metabolism, miRNA are non-coding short molecules (± 22 nucleotides) that regulate the biological activity of mRNA by binding to the 3' untranslated regions, thus either blocking translation or facilitating the degradation of the respective target mRNA. Current knowledge suggests that miRNA's are expressed in tissue-specific manner. Thus differences in miRNA signature were found for miRNA-335 (ratio bmMSC vs. DF, 44-fold). Thus measuring miRNA-335 in cells may help for quality control of bmMSC. It remains to be determined if the expression of miRNA's may help to discriminate DF from MSC of different sources including adipose tissue, peripheral blood, placenta, amnion membrane, etc. This is of importance as MSC maintain a gene expression signature depending on the positioning of their precursor cells in the anlagen during embryonic development [53]. This miRNA-335 binds to the mRNA encoding a hydrolase named MEST or PEG1. Dysregulation of MEST/PEG1 was found in cancer cells and genetic deletion of miRNA-335 is very common in breast cancer [54].

Raman Spectroscopy for non-contact discrimination of cells

Very recently, a promising study pioneered a novel method to discriminate even closely related but still different types of cells: Raman spectroscopy [55]. Hereby, live cells are activated via a high-power infrared laser beam. The laser photons interact with molecules and lift them on high-energy states. When the molecule relaxes it emits photons in any direction (scatter), which are detected by sensitive photomultipliers or CCD cameras. The intensity and wavelength of scattered light is measured. The Raman spectroscopy proved to be a very sensitive method as 20 -30 cells per analysis were sufficient to generate a spectrum [55,56]. In addition, a single measurement took less than 2 minutes for data acquisition and did not require time-consuming preparations beforehand. Flow cytometry for instance requires several steps in preparation of the data acquisition: incubation of cells with antibodies, washing and possibly even addition of detection reagents. For characterization of MSC we routinely stain $1x10^5$ cells and acquire data from $5x10^4$ events. In addition, MSC stained with antibodies often can't be immediately processed for a clinical application. Therefore additional steps for removal of antibodies have to be added when MSC will be applied to humans after flow cytometry or fluorescence activated cell sort (FACS). In contrast, Raman spectroscopy does not require any manipulation of the cells to be investigated and, as far as we can say today, the infrared laser-based methods seem not to harm cells.

The Raman spectra have also been utilized to investigate live cells in tissues [55]. This technique therefore may allow monitoring of behavior of MSC *in situ* during wound healing. Right now we are still at the beginning of this development. Therefore we are convinced that this Raman technique will soon cross the threshold in the field of regenerative medicine and tissue engineering.

Stemness of MSC

The above mentioned experts in grand rounds [30] and consensus conferences [27,28] stated that the triple lineage differentiation potential of MSC was an important hallmark to better define the fibroblast-like MSC. MSC was the acronym for mesenchymal *stem* cells [28]. The terminus "stem cell" is in use for unspecialized cells that can differentiate into more specialized cells of one type or several types or lineages. A stem cell replaces cells that die or are lost in the body and retains lifelong proliferation and self-renewal. In *bona fide* MSC expression of factors characteristic *"stemness"*, i.e. cell proliferation and differentiation such as IGF1, IGFBP4, AKT3, STAT1, STAT4,

SMAD3, FZD7, DKK3 were detected [57]. At the same time these cells proliferated well *in vitro*. But this concept of a stem cell maintaining full "*stemness*" at continuous proliferation is not supported by investigation of the hematopoietic stem cell niche and HSC therein [9]. The true hematopoietic stem cell (HSC) divides *in situ* very slowly, but expresses telomerase at high levels to keep the chromosomal ends complete [58] (see below). From this slow proliferating true HSC, rapidly dividing progenitor cells are derived which have already lost the full potential of the HSC. *In vitro* a slow proliferating stem cell will be lost between the proliferating progenitors cells, unless we utilize a reagent, antibody, ligand, feeder cell, or any tool to first select the stem cell specifically and then maintain this cell in culture, if possible as a true clone. Such a tool, reagent, antibody or alike will be beneficial in discriminating MSC or even subsets of MSC from DF. It also will be helpful to better localize the true MSC in its niche or niches *in vivo*. It also may help to define MSC satisfactorily for authorities {e.g., EMA (www.ema.europa.eu), FDA (www.fda.gov)} involved in approval of cell-based therapies.

In contrast to true stem cells, proliferating MSC display signs of senescence after repeated rounds of passaging *in vitro* and fail to express telomerase at substantial levels [59-61]. Furthermore, clones of permanent cells lines reportedly developed from MSC after long term expansion [62]. But they were shown to be contaminants overgrowing the original MSC population, at least in some cases [63].

Another problem with the concept of "*stemness*" of MSC is observed with regard to the differentiation potential. MSC from different sources display a distinct differentiation potential. For instance, placenta-derived MSC do not generate osteoblasts efficiently *in vitro* [26] and umbilical cord-derived MSC will not become adipocytes effectively [64]. Others reported a preferential chondrogenic differentiation potential in MSC derived from synovial membranes [65], but a more detailed study revealed later that the *in vitro* chondrogenically differentiated MSC failed to generate a stable cartilage tissue at ectopic sites [66]. Therefore the differentiation potential of MSCs depends very much on the source of cells and the experimental conditions as well. Here, therefore, the aforementioned position signature of MSC may come into play [53]. Moreover, MSC change their differentiation potential *in vitro* [17]. Therefore an important criterion to name a cell *stem cell* is not really fulfilled by MSC: long-term self-renewal and full differentiation competence at the same time. Consequently at the present time the term mesenchymal *stromal* cell is preferred.

On the other side, fibroblasts were shown to have a differentiation potential and generation of adipocytes, chondrocytes and osteoblasts [48,67] or adipocytes and osteoblasts [68] was induced from human DF (hDF). Moreover, both populations, the adipose tissue-derived MSC and the DF expressed factors found in embryonic stem cells [52] and hDF seem to express KLF4 at high levels compared to MSC [67]. Therefore it seems that hDF share expression of many cell surface proteins with MSC, including the markers commonly utilized for MSC characterization [27]. In addition, hDF display a tri-lineage differentiation potential and are capable of generating colonies *in vitro* [48,67,68]. Although MSC and DF were studied intensively with cells from a variety of species, the differences between these two types of cells are not yet fully evident.

Conclusion

At present, a simple guide as how to best discriminate human mesenchymal stromal cells derived from bone marrow, adipose or other tissues from human dermal fibroblasts or fibroblast harvested from other source is not at hand. To name a cell a MSC, it must fulfill the inclusion criteria [27,28,30] and maintain a differentiation potential at least for a few cycles of cell division during expansion *in vitro*. But fibroblasts share many features with "true" MSC, including a basic differentiation capacity.

Note that the limited differentiation capacity of some fibroblasts or some MSC will be advantageous in a given clinical context: A prominent osteogenic differentiation for instance and deposition of a calcified extracellular matrix by bmMSC is cumbersome when soft tissue such as adipose or muscular tissue needs regeneration or repair. In this context autologous dermal fibroblast or placenta-derived MSC yield advantages. In the end, the definitive discrimination between MSC and fibroblasts seems to be a purely academic matter. It seems more important what a given cell will do *in vitro* or *in vivo* and for which particular experimental or clinical need a given cell really is the best component. But in the context of regenerative medicine and for cell-based therapies, a defined tool to discriminate MSC from other cells would be of tremendous progress. We therefore entertain in our laboratory studies investigations using both MSC and DF and utilize cells from different niches or tissues, expand the cells under conditions compatible to the standards required for clinical application (i.e., good medical procedures, GMP) in order to find the best type of cell for a given purpose.

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