

Biological Similarities Between Mesenchymal Stem Cells (Mscs) and Fibroblasts

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Abstract

Fibroblasts have been shown to possess the capacity to differentiate into follicular dendritic cells in lymph nodes and into myofibroblasts in wounded tissues. Here, we demonstrate that foreskin-derived fibroblasts also have the potential to differentiate into adipocytes and osteoblasts under cell culture, developed for the differentiation of bone marrow-derived mesenchymal stem cells (BM-MSc).

Keywords: Mesenchymal stem cells; Microarray analysis; Fibroblasts differentiation

Abbreviations: MSC: Mesenchymal Stem Cells; BM-MSc: Bone Marrow-Derived Mesenchymal Stem Cells; ECM: Extracellular Matrix; FACS: Flow Cytometric Analysis; PBS: Phosphate Buffered Saline; DMEM: Dulbecco's Modified Eagle Medium; BAP: Bone Alkaline Phosphatase

Introduction

Bone marrow-derived mesenchymal stem cells have the capacity to differentiate into several cell types [1,2]. Fibroblasts are referred to as mature counterpart of MSCs [3], even though they can differentiate into follicular dendritic cells [4,5] and myofibroblasts [6], the latter type of cells play an important role in wound healing. In addition, fibroblasts contribute to synthesis and remodeling of extracellular matrix (ECM) proteins and are able to respond to external stimuli by production of several cytokines, e.g. CCL2, IL6 [7], and IL 8 [8]. Fibroblast-derived cytokines have an impact on the immune system in Graves' disease and tumor tissue [7-10].

In this study we show that the fibroblasts exhibit a similar surface protein expression profile when compared to MSC. Furthermore, we demonstrate expression of 4-prolylhydroxylase and fibronectin in both cell types. Microarray analysis of gene expression also revealed many similarities between MSCs and fibroblasts. These data suggest that MSCs and tissue-resident fibroblasts are closely related, if not even representing two variants of the same cell.

Material and Methods

Isolation and cell culture of MSCs and fibroblasts

Bone marrow samples were collected from healthy donors after informed consent. MSCs were isolated via their plastic adherence. Briefly, mononuclear cells were isolated from bone marrow by a Ficoll-Paque Plus (Amersham Biosciences AB, Uppsala, Sweden). After one day the non-adherent cells were removed by replacing the medium.

Fibroblasts were generated according to a protocol by Rodemann and Bayreuther (1984).

Flow cytometric analysis

For analysis, Fc receptors were blocked with FcR Blocking Reagent (Miltenyi Biotec GmbH) and incubated with antibodies: fluorescein-

(FITC) conjugated CD44 (clone L178, Pharmingen, BD Bioscience, San Diego, CA, USA), FITC-conjugated CD105 (clone CLB-HEC19, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam, The Netherlands), FITC-conjugated CD45 (clone 5B1, Miltenyi Biotec GmbH), phycoerythrin- (PE) conjugated CD29 (clone MAR4), PE-conjugated CD73 (clone AD2), PE-conjugated CD166 (clone 3A6) (all from BD Pharmingen), PE-conjugated CD133 (clone 293C3), PE-conjugated CD14 (clone TÜK 4), and PE-conjugated CD34 (clone AC136) (all from Miltenyi Biotec GmbH). Isotype controls were purchased from Pharmingen, BD Bioscience. FACS analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Dead cells were excluded via propidium iodide staining and data analysis was performed with BD CELLQuest software.

Induction of osteoblast and adipocyte differentiation

Induction of osteoblast and adipocyte differentiation was achieved using NH Osteodiff Medium and NH Adipodiff Medium (both from Miltenyi Biotec GmbH). Each differentiation experiment was conducted with fibroblasts from five different donors (passage 2-6, 19-69 days in culture, median 43 days). MSCs from eight individual donors were used as positive control for the adipocyte differentiation (passage 2-5, 8-34 days in culture, median 22 days). For the osteoblast differentiation MSC from ten donors were used (passage 1-5, 6-40 days in culture, median 26 days). After ten days in NH Osteodiff Medium, osteoblasts were identified by their expression of alkaline phosphatase using the Fast BCIP/NBT Kit (Sigma Aldrich, Steinheim, Germany) according to manufacturer's instructions. Adipogenic differentiation was confirmed by staining lipid droplets after 21 days culture in NH Adipodiff Medium. Therefore, cells were fixed with methanol for 10min at -20°C and lipid droplets were stained with

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Accepted October 18, 2010; Published October 18, 2010

Citation: Ghodsizad A, Voelkel T, Moebius JM, Gregoric I, Bordel V, et al. (2010) Biological Similarities Between Mesenchymal Stem Cells (Mscs) and Fibroblasts. J Cytol Histol 1:101. doi:10.4172/2157-7099.1000101

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a 0.3% Oil red O (Sigma Aldrich) solution (isopropanol/ water (6:4)) for 20min at room temperature, washed with distilled water and examined under a microscope.

Immunocytochemistry

MSCs and fibroblasts were grown on glass cover slips (Menzel, Braunschweig, Germany). After washing twice, unspecific binding sites were blocked and cell membranes were permeabilized by incubation with PBS/5% normal goat serum (Vector Laboratories, Burlingame, CA, USA)/ 0.5% TritonX-100 (Sigma-Aldrich). Then cells were incubated with anti-fibronectin monoclonal antibody (1:20, clone EP5, RDI Division of Fitzgerald Industries Intl, Concord MA, USA) for 1 hour at 4°C. After washing twice with PBS/ 5% normal goat serum, bound primary antibody was detected by incubating with rat anti-mouse IgG,-Alexa 488 (clone CLX56, labeled using the Alexa Flour 488 Protein Labeling Kit, Molecular Probes, Invitrogen, Karlsruhe, Germany) for 1 hour at 4°C. After washing again with PBS/ 5% normal goat serum, nuclei were stained using Toto3 (1:1000, 15min, RT, Molecular Probes). Then cells were washed carefully with PBS, mounted with Fluoromount G (Southern Biotech, Birmingham, USA) and analyzed with a Leica DMIRE2 confocal microscope (Leica Microsystems, Wetzlar, Germany) using Leica Confocal Software.

Western blot analysis

Western Blot analysis was performed with cell lysates of two different fibroblast and two different MSC samples. Lysates of peripheral blood mononuclear cells were used as negative and cell lysates of the cell line NT2 as positive control. Before electrophoretic separation by SDS-PAGE (Tris-Glycine 10-20%, Novex, Invitrogen), the lysates equivalent to 2×10^4 cells were boiled for 5min with reducing electrophoresis sample buffer. After SDS-PAGE, the proteins were blotted onto a cellulose nitrate membrane, which was then blocked with phosphate buffered saline (PBS) containing 0.1% Tween 20 (Sigma Aldrich) and 5% defatted milk for 1h. Then the membrane was incubated with an anti-4-prolylhydroxylase antibody (clone 3-2B12, 1:100, Acris Antibodies, Hiddenhausen, Germany) for 1h at room temperature, which was detected with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:10000, Jackson Immuno Research Laboratories, Cambridgeshire, UK) and the ECL Plus Western Blotting Detection System (Amersham, Buckinghamshire, UK) according to manufacturer's instructions.

Microarray analysis

RNA was prepared from four independent samples of cultivated human foreskin fibroblasts and human MSCs, respectively, using the Nucleospin-II-RNA-Extraction-Kit (Macherey&Nagel) at passage four (MSCs: passage 1-3, 20-40 days in culture, median 29 days; fibroblasts: passage 4 and 5, 28-48 days in culture, median 37 days).

Linear amplification of RNA was performed using a modified protocol of a method previously describe [11,12]. One microgram of aRNA from each sample was labeled by reverse transcription with Cy5 (individual samples) and Cy3 (common reference control) fluorescence, respectively. Labeled samples were then hybridized on Human Stem Cell PIQOR™ Microarrays (Miltenyi Biotec), which contain cDNAs of 930 genes spotted as four-fold replicates on different positions of the array. Hybridization, scanning and data analysis were performed as described in detail by Bosio et al. (2002) [13]. Briefly, image capture and signal quantification of hybridized PIQOR™ microarrays were performed with the ScanArrayLite4000 Microarray Scanner (Packard Biosciences, Wellesley, MA, USA) and ImaGene software Version 5.0 (BioDiscovery, Los Angeles, CA, USA).

Local background was subtracted from the signal to obtain the net signal intensity and the ratio of Cy5/Cy3. Subsequently, the mean of the ratios of four corresponding spots representing the same cDNA was computed. The mean ratios were normalized using the Median method [14]. Only genes displaying a net signal intensity 2-fold higher than the mean background were used for further analysis. Two class unpaired significance analysis of microarray (SAM) [15] was performed using the 2-fold change criterion and a false discovery rate of 0,001%. Only those genes present in all experiments were subjected to SAM.

Reverse transcriptase PCR

Total RNA of 1×10^6 MSCs and of 1×10^6 fibroblasts of three independent samples each was extracted using the NucleoSpin RNA II Kit (Macherey & Nagel, Dueren, Germany) according to the manufacturer's instructions at passage four. cDNA was synthesized in a 30µl reaction mixture containing 300 U SuperscriptII, 1x first strand buffer (0.3µM, Invitrogen), dNTP (200µM each, PEQLAB, Erlangen, Germany), Oligo(dT)18 (5µg) and (N6) random hexamere (0.2µg, both Metabion, Martinsried, Germany). PCR was performed in a 50µl reaction mixture containing 1.25 units Taq polymerase, 1x reaction buffer, 2mM MgCl₂ (Fermentas International Inc., Burlington,

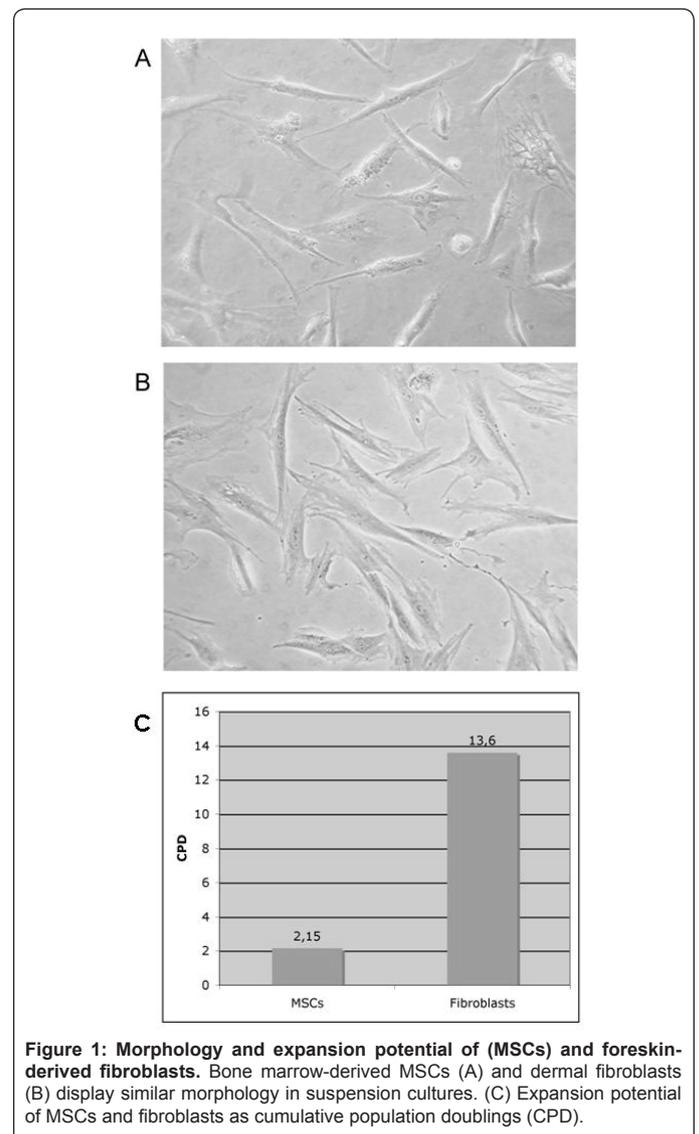


Figure 1: Morphology and expansion potential of (MSCs) and foreskin-derived fibroblasts. Bone marrow-derived MSCs (A) and dermal fibroblasts (B) display similar morphology in suspension cultures. (C) Expansion potential of MSCs and fibroblasts as cumulative population doublings (CPD).

Ontario, Canada), 200µM of each dNTP (PEQLAB, Erlangen, Germany) and 200nM primers (Metabion). cDNA concentrations were normalized to the β-actin housekeeping gene. The following primers were used: β-actin (forward: gagaagatgaccgatcatgt, reverse: catctcttctcgaagtccag), THY 1 (forward: gagaataccagcagttccaccatc, reverse: aggatctctgcactggaacttgag) and nestin (forward: cagcgttggaacagaggttg, reverse: gctggcacaggtgtctcaag). The cycling conditions comprised an initial denaturation of 5min at 94°C and 30 cycles of 1 min at 94°C, 1min at 60°C and 1min at 72°C followed by a final elongation of 10min at 72°C.

Results

Morphological similarities and expansion potential of MSCs and fibroblasts

MSCs and foreskin-derived fibroblasts were maintained in DMEM supplemented with 20% FCS. Both cell types showed the same morphology at passage four as depicted in Figure 1A and 1B. Determination of the expansion potential by calculation of cumulative population doublings after 20 days of culture revealed that skin derived fibroblasts were better expandable (Figure 1C).

Analysis of surface marker expression

Flow cytometry analysis was performed in order to determine the surface protein expression of several known MSC markers on foreskin-derived fibroblasts (Figure 2). MSCs were found to be positive for CD29, CD44, CD73, CD105 and CD166 (Figure 2A) while they did not express the hematopoietic markers CD14, CD34, CD45 and CD133 (Figure 2B). Fibroblasts showed a similar staining pattern and there was no significant variation of surface marker expression among different samples.

Fibroblasts differentiate along different mesenchymal lineages

To examine the multilineage differentiation capacity of skin-derived fibroblasts, cells were differentiated towards the adipogenic and the osteogenic lineage using cell culture regimens established

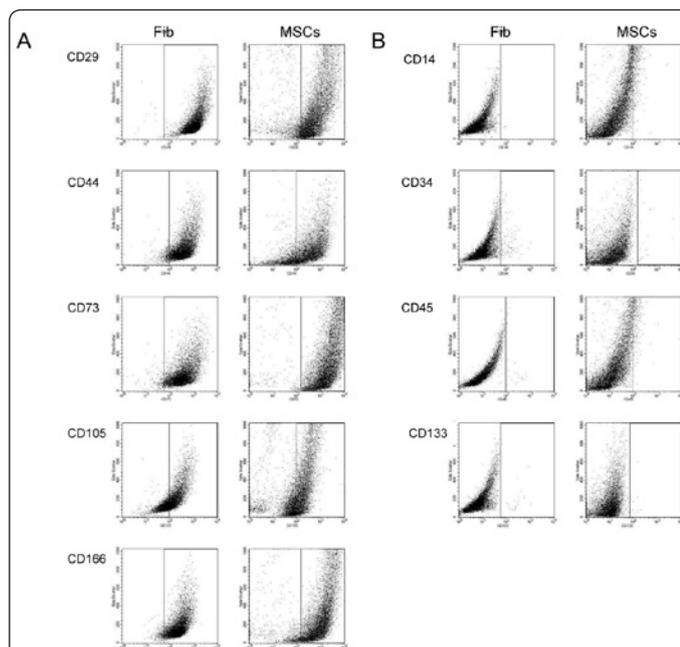


Figure 2: Phenotypical comparison of MSCs and fibroblasts. Representative flow cytometric analysis revealing highly similar antigen expression patterns.

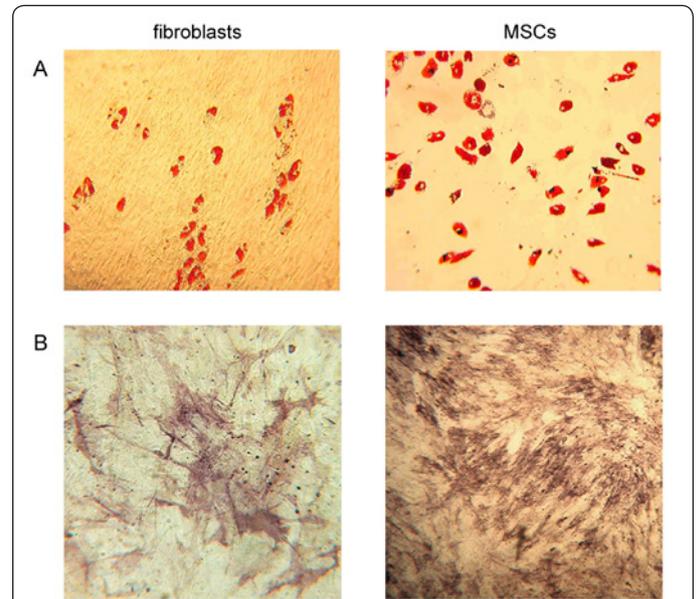


Figure 3: Analysis of the differentiation capacities of foreskin-derived fibroblasts. Differentiation of the dermal fibroblasts; (A) Adipogenic differentiation (B) Osteogenic differentiation.

for the differentiation of MSC. After adipogenic induction of fibroblasts (n=5), adipocytes were identified by their accumulation of triglyceride droplets, which can be detected using Oil red O staining. Fibroblasts from four tested donors showed lipid accumulation (80%, Figure 3A). MSCs from eight donors were used as positive control and seven showed lipid accumulation (87.5%). In order to investigate the osteogenic differentiation potential of the fibroblasts, cells were analyzed for their expression of alkaline phosphatase, an enzyme that is involved in bone matrix mineralization. Cells from four out of five fibroblast donors were found to express this enzyme after 10 days of culture (80%, Figure 3B). MSCs from ten donors served as positive control and 100% could be differentiated into osteoblasts. These data show that foreskin-derived fibroblasts are able to differentiate along different mesenchymal lineages.

Expression of fibroblast marker proteins by MSCs

The findings mentioned above suggest a close developmental relationship between fibroblasts and MSCs. To confirm this hypothesis, fibroblasts as well as MSCs were further examined regarding their expression of the fibroblast marker proteins 4-prolylhydroxylase and fibronectin. 4-prolylhydroxylase is an intracellular enzyme required for the synthesis and formation of all of the 20 known types of collagen. It enables the formation of stable triple helical structures by modification of prolyl residues during post-translational modification of collagen [16,17]. Lysates of fibroblasts and MSCs were tested for the expression of 4-prolylhydroxylase using Western Blot analysis. As can be seen in Figure 4A, 4-prolylhydroxylase could be detected in all lysates tested. Expression of fibronectin, an extra-cellular matrix protein, was verified by immunocytochemical staining. MSCs as well as fibroblasts were found to express this protein (Figure 4B). These data strongly suggest that fibroblasts as well as MSCs produce the extracellular matrix proteins collagen and fibronectin.

Micoarray analysis

To further investigate the fibroblast and MSC marker profile, microarray analysis employing the human stem cell PIQR™

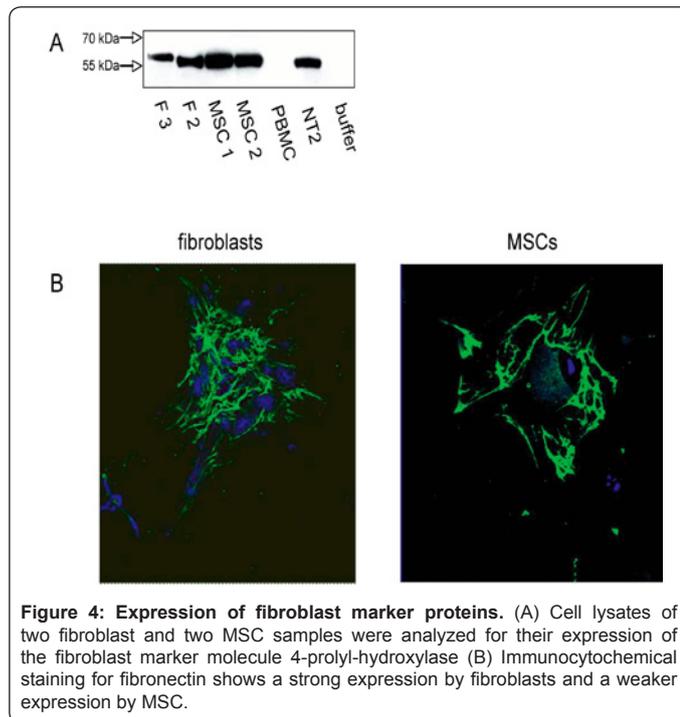


Figure 4: Expression of fibroblast marker proteins. (A) Cell lysates of two fibroblast and two MSC samples were analyzed for their expression of the fibroblast marker molecule 4-prolyl-hydroxylase (B) Immunocytochemical staining for fibronectin shows a strong expression by fibroblasts and a weaker expression by MSC.

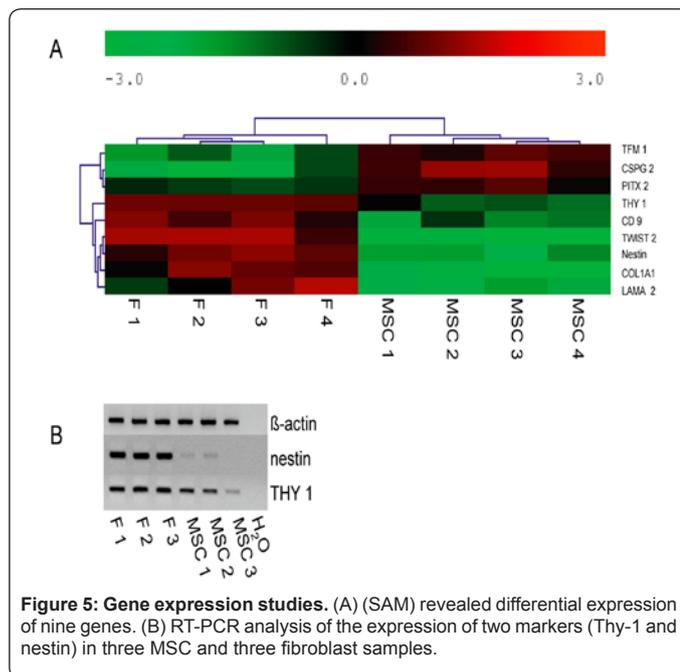


Figure 5: Gene expression studies. (A) (SAM) revealed differential expression of nine genes. (B) RT-PCR analysis of the expression of two markers (Thy-1 and nestin) in three MSC and three fibroblast samples.

microarray was performed. This microarray contains 930 probes spotted in quadruples, which were selected for relevance in stem cell research. Labeled cDNA of four fibroblast and four MSC samples was hybridized against a standard, which was produced by pooling total RNA of all samples prior to labeling and cDNA synthesis. Several genes involved in bone formation were found to be expressed in fibroblasts and MSCs at similar levels, e.g. BMP-2, Runx-2, Smad-1, osteocalcin precursor BGLAP, and ITGA9, which is part of the osteopontin receptor (data not shown). Besides Bmp-2, other members of the TGF- β superfamily (GDF2, GDF3, GDF8) and Smad2 that is part of the TGF β 2 pathway were expressed at equal levels.

Additionally, expression of several genes involved in NF- κ B signaling (CHUK, RELA, PRKCCQ and TNFRSF14) appeared to be similar in MSCs and fibroblasts.

Significance analysis of microarrays (SAM) identified only nine genes, which showed a difference in expression levels of > 2-fold (Figure 5A). Three of these genes showed higher expression in MSCs than in fibroblasts; tropomyosin 1 alpha chain (TFM 1), the ECM protein versican (CSPG2), and the transcription factor PITX2. The six other genes, which showed higher expression levels in fibroblasts, include the stem cell marker nestin, the ECM molecule collagen alpha 1 (COL1A1), the alpha 2 chain of laminin (LAMA2), Thy-1 (CD90), CD9, and Twist2 (Dermo1). Differential expression of nestin and THY-1 was verified independently by RT-PCR (Figure 5 B).

Discussion

Similarities between fibroblasts and MSCs were further studied by gene expression analysis. Microarray analysis revealed that several genes, such as BMP-2, Runx-2, Smad-1, osteocalcin precursor BGLAP, and ITGA9, which are involved in bone formation, are expressed at equal levels. Bmp-2 has been shown to lead to the expression of the transcription factor Runx-2 through the second messenger Smad-1 [18,19]. Runx-2 then activates osteocalcin and bone alkaline phosphatase (BAP). Osteocalcin prevents mineralization until enough phosphate has been accumulated. Then increased inorganic phosphate concentrations induce the expression of osteopontin [20,21]. Osteopontin has also been connected with NF- κ B expression and like NF- κ B, has been suggested to mediate mechanical stress signaling [22,23]. Interestingly, additional genes involved in NF- κ B signaling and other members of the TGF- β superfamily besides BMP-2 were found to be similarly expressed in MSCs and fibroblasts.

Only 9 genes could be identified which were differentially expressed by both cell types. Three of these genes, namely tropomyosin 1 alpha chain, PITX2, and versican showed a higher expression level in MSCs. Tropomyosin 1 alpha chain is expressed in smooth muscle cells where it is involved in the contractile system, but it also plays a role in non-muscle cells as cytoskeleton component. PITX2 has been shown to regulate procollagen lysylhydroxylase expression [24]. Hydroxylation of lysine residues directs the collagen cross-linking pattern and provides attachment sites for carbohydrate units. Lysylhydroxylase has been shown to mediate collagen synthesis in dermal fibroblasts [25]. It has also been reported that fibroblasts express versican [26,27].

6 genes (nestin, collagen alpha 1, the alpha 2 chain of laminin, Thy-1 (CD90), CD9, and Twist2) showed higher expression levels in fibroblasts. Thy-1 is known to be expressed on a subset of fibroblasts and has been suggested to be implicated in the differentiation of myofibroblasts [28]. CD9 is involved in cell adhesion and appears to promote muscle cell fusion and to support myotube maintenance [29,30], while Twist2 has been reported to maintain cells in a pre-osteoblast phenotype [31]. CD9 has been shown to be also expressed by MSCs [32]. PCR revealed expression of nestin and Thy-1 by MSCs as well. Expression of nestin by MSCs has already been demonstrated by other groups [32,33,34].

Nestin, one of the intermediate filament components of a cell, is a marker of neural stem cells or progenitor cells [35]. During development, nestin is expressed in migrating and proliferating cells [36]. In contrast, in adult cells and tissues, expression of nestin is mainly restricted to areas of regeneration [37-41]. It is widely believed that nestin indicates multilineage differentiation and high regenerative potential of a cell reviewed by Wiese et al. 2004 [42].

One of the most important physiological functions of fibroblasts is the wound repair and migration. Hence, expression of the stemness marker nestin by fibroblasts suggests additional repair mechanisms via differentiation. This hypothesis is supported by a study, clearly showing that nestin-positive fibroblast-derived cell lines can display characteristics of embryonic stem cells [43].

Several *in vivo* studies have demonstrated that bone marrow cells can give rise to fibroblasts and myofibroblasts [44-48]. It has also been shown that transplanted MSCs can regenerate dermal structures after wounding [49-50]. A study by Francois et al. [51], where MSCs were transplanted into irradiated mice, also supports the concept that MSC-like cells in the bone marrow or MSC precursors might be able to migrate and contribute to tissue repair by replacing lost fibroblasts and myofibroblasts, at least in case of injury. These studies imply a strong functional overlap between MSCs and local tissue fibroblasts. MSC-like cells have been isolated from connective tissues of various organs [52-58]. Our findings indicate that these "MSCs" in fact might be local tissue fibroblasts that can differentiate into different mesenchymal lineages.

In the dermis, two sources of stem cells have been described: bulge area stem cells, which contribute to hair growth [54-55] and skin-derived precursors (SKPs) [56-59]. SKP cultures can be established by culturing dermal cell suspensions in neurogenic media. Under these conditions SKPs form spheres, which can be passaged. Because SKPs can also be generated from foreskin, which does not contain hair follicles, they cannot represent bulge area-derived stem cells. Our hypothesis is that, like connective tissue-derived MSCs SKPs originate from dermal fibroblasts. The results presented here indicate that dermal fibroblasts show a similar differentiation potential as bone marrow MSCs and consequently, might also be suitable candidates in these clinical situations.

Acknowledgements

We would like to thank Kathrin Godthardt and Daniela Micheli for technical assistance.

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