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Distinct Gene Expression Profile of Human Mesenchymal Stem Cells in Comparison to Skin Fibroblasts Employing cDNA Microarray Analysis of 9600 Genes

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Broad differentiation capacity has been described for mesenchymal stem cells (MSC) from human bone marrow. We sought to identify genes associated with the immature state and pluripotency of this cell type. To prove the pluripotent state of the MSC, differentiation into osteocytes, adipocytes, and chondrocytes was performed in vitro. In contrast, normal skin cells did not harbor these differentiation abilities. We compared the expression profile of human bone marrow MSC with cDNA from one primary human skin cell line as control, using a cDNA chip providing 9600 genes. The identity of all relevant genes was confirmed by direct sequencing. Data of gene array expression were corroborated employing quantitative PCR analysis. About 80 genes were differently expressed more than threefold in MSC compared to mature skin fibroblasts. Interestingly, primary human MSC were found to upregulate a number of genes important for embryogenesis such as distal-less homeo box 5, Eyes absent homolog 2, inhibitor of DNA binding 3, and LIM protein. In contrast, mesenchymal lineage genes were downregulated in MSC in comparison to skin cells. We also detected expression of some genes involved in neural development, indicating the broad differentiation capabilities of MSC. We conclude that human mesenchymal stem cells harbor an expression profile distinct from mature skin fibroblast, and genes associated with developmental processes and stem cell function are highly expressed in adult mesenchymal stem cells.

Key words: Mesenchymal stem cells; Microarray; Differentiation; Gene expression profile

MESENCHYMAL stem cells (MSC) from human bone marrow are one of the most accessible adult stem cells described to date. Mesenchymal stem cells have first been described as fibroblasts colony-forming cells (24,25). Due to their differentiation abilities they recently have been termed marrow stromal cells (69), mesodermal progenitor cells (MPC) (73), or multipotent adult progenitor cells (MAPC) (31). MSC can be differentiated in vitro into osteocytes, chondrocytes, adipocytes, myocytes, endothelial cells, and hematopoesis-supporting stroma (68,73). They seem to contribute to most, if not all, somatic cell types, when injected into early blastocysts or into xenogenic embryos (31,43). In contrast, skin fibroblasts, the ma-

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ture counterpart of MSC, do not harbor these broad differentiation abilities (68). Yet, little is known about genes and signal transduction pathways involved in the maintenance of pluripotency or its loss.

Microarray analysis is a useful screening technique for gene expression profiles. Therefore, we compared the expression profile of MSC cDNA obtained from human bone marrow of four healthy donors with that from primary human skin fibroblasts derived from healthy donors as control. Employing this technique we were interested to investigate gene expression profiles of MSC that could be associated with the pluripotent state and differentiation capacity of MSC.

MATERIALS AND METHODS

Mesenchymal Stem Cell Culture

The culture methods of Pittenger (68) were employed here with minor modifications. Spongiform bone fragments from hip replacement operations were taken from otherwise healthy volunteers after written informed consent at one institution (Philipps-University, Marburg). The protocol had been approved by our local Ethics Committee. Cells were processed within 12 h after collection. Specimens were thoroughly minced, passed through a 70-μm filter mesh, and subsequently mononuclear cells (MNCs) were obtained by Ficoll™ (Amersham Pharmacia, NJ, USA) density gradient centrifugation, washed with phosphate-buffered saline (PBS) (Biochrom, Berlin, Germany), and resuspended in Dulbecco's modified medium (DMEM) with low glucose and glutamine (PAA, Linz, Austria) with 10% fetal calf serum (FCS) from selected lots (Stem Cell Technologies, Vancouver, Canada) and 1% penicillin/streptomycin. Cells were plated at a concentration of 200,000 cell/cm^2 in plastic flasks. Medium was replaced after the first 24 h and then every 3–4 days until fibroblasts grew confluent. Cells were then collected by treatment with trypsin/EDTA solution (Roche, Basel, Switzerland) for 10 min at 37°C, rinsed with medium, and split for three new culture dishes of the same size. With every splitting and exchange of a culture dish the passage number was increased.

To ensure purity of MSC, flasks were washed two times thoroughly with PBS and the medium was replaced within 24 h. With this technique contamination of hematopoietic stem cells, macrophages, or endothelial cells could be avoided effectively as proved by flow cytometric antigen expression analysis. For staining of surface molecules, 10^5 to 10^6 cells were incubated in 100 μl PBS with 10 μl of fluorochrome-labeled monoclonal antibody for 20 min at 4°C in darkness. Then cells were washed with PBS, resuspended in 300 μl PBS, and immediately submitted to FACS analysis (FACScan, Becton Dickinson, San Jose, CA, USA). For the CD105 antibody (PharMingen, San Jose, CA, USA) indirect staining was performed with incubation of the primary antibody for 20 min at 4°C, two times washing, staining for 10 min with the goat anti-mouse PE secondary antibody (PharMingen), washing with PBS, and resuspension in 300 μl PBS for further analysis. The following antibodies were employed: CD45-FITC (PharMingen), CD90-FITC (PharMingen), CD34-PE (PharMingen), CD14-PE (Becton Dickinson), CD31-PE (Beckmann-Coulter, Krefeld, Germany), CD44-FITC (Becton Dickinson), IgG-FITC and IgG-PE (Becton Dickinson).

Cloning efficiency of MSC had been investigated under different conditions (different FCS lots, different media, use of conditioned media). MSC culture could be maintained for more than 20 passages (1 year) and efficient cloning was proved by CFU-fibroblast (CFU-F) formation even after 7 months of in vitro culture. We used MSC cells before the third passage for microarray experiments and for further differentiation procedures.

Cell Cultures of Skin Fibroblasts

Small pieces of skin from the patients undergoing hip replacement were cut into tiny pieces and then digested with 0.2% collagenase (Roche, Basel, Switzerland) for 15 min at 37°C, followed by 0.1% trypsin incubation (Invitrogen, Grand Island, NY, USA) for 30 min at 37°C. The tissue was triturated briefly and then passed through a 70-μm filter. Cells were collected by centrifugation and then resuspended in DMEM medium with 10% FCS (Gibco BRL, Grand Island, NY, USA) and 1% penicillin/streptomycin. Medium was replaced every 3–4 days until the cells grew confluent. Skin fibroblasts were harvested by treating with trypsin/EDTA solution and were split into three portions for three new culture flasks. For microarray experiments and differentiation skin fibroblasts were harvested at the second passage.

Osteogenic Differentiation

For osteogenic differentiation the cells were harvested as described above and were allowed to adhere within 24 h in standard medium in humidified air of 5% CO_2 at 37°C. Subsequently, standard medium was replaced through osteogenesis induction medium with 10% FCS from selected lots (Stem Cell Technologies), DMEM low glucose with glutamine, 0.1 μM dexamethasone, 0.05 mM ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate (all obtained from Sigma, Taufkirchen, Germany). Medium was replaced every 3–4 days. After 10–14 days of cul-

ture, cells were harvested for FACS analysis as described below. Slides were fixed with acetone/methanol (1:1) at −20°C for 5 min to perform hematoxylin & eosin (H&E) and von Kossa staining of the extracellular calcium matrix as described (Poetics/Bio Whittaker/Cambrex; Instructions for use of mesenchymal stem cells) (68).

Chondrogenic Differentiation

To induce chondrogenic differentiation 2.5×10^5 MSC or skin fibroblasts were cultured in 15 ml conical polypropylene tube chondrogenesis induction medium consisting of the following ingredients: DMEM high-glucose medium, 0.1 μM dexamethasone, 1 mM sodium pyruvate, 0.17 mM ascorbic acid-2-phosphate, 0.35 mM proline, 6.25 μg/ml bovine insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 5.33 μg/ml linoleic acid, 1.25 μg/ml BSA, and 0.01 μg/ml TGF-β3 (all obtained from Stem Cell Technologies). Cells were cultured in humidified air of 5% CO_2 at 37°C with a medium change every 3–4 days and were harvested after 21 days in culture. Only MSC after induction of differentiation but not the skin fibroblast or the control cells, which were pelleted in the same way but cultured in standard medium, formed small solid aggregates due to generation of surrounding solid matrix. The small cartilage piece and the loose aggregates from skin fibroblast differentiation and controls were embedded in paraffin for microdissections and subsequently stained with Alzian blue and nuclear fast red stain to characterize the proteoglycan extracellular matrix and nuclei as described.

Adipogenic Differentiation

For adipogenic differentiation the cells were cultured in standard medium. After confluence they grew 3–4 days in standard medium and subsequently the medium was replaced through adipogenic induction medium for 3 days. The adipogenic induction medium consisted of DMEM high-glucose medium (PAA), 10% FCS (Stem Cell Technologies), 1% penicillin/streptomycin, 1 μM dexamethasone, 0.2 mM indomethacin, 0.01 mg/ml insulin, 0.5 mM 3-isobutyl-1-methyl-xanthine (Sigma, Taufkirchen, Germany). After 3 days the adipogenic induction medium was replaced through adipogenic maintenance medium, consisting of DMEM high-glucose medium, 10% FCS from selected lots, 1% penicillin/streptomycin, 0.01 mg/ml insulin. This treatment of medium replacement was repeated three times. The cultures were maintained in adipogenic maintenance medium for 1 week. After this time the slides were fixed and stained with H&E and Sudan black B to visualize the lipid vacuoles. The cells were harvested, RNA was extracted, and RT-PCR was performed to analyze the expression of peroxisome proliferator-activated receptor-γ2 (PPARγ2) as described.

Flow Cytometric Analyses of Differentiation

The cells were harvested with trypsin/EDTA, washed by centrifugation with PBS, and 10^5 to 10^6 cells were incubated in 100 μl PBS with 10 μl fluorochrome-labeled monoclonal antibody for 20 min at 4°C in the dark. Subsequently cells were washed and resuspended in 500 μl PBS and FACS analysis was immediately performed using a FACScan analyser (Becton Dickinson). For the anti-bone sialoprotein antibody (Calbiochem, San Diego, CA, USA) indirect staining was performed with 10 μl primary antibody. After incubation for 20 min at 4°C and two times washing with PBS labeled cells were stained with secondary FITC goat anti-mouse antibody (PharMingen) for 10 min. They were washed two times in PBS and analyzed immediately. The following antibodies were used: CD45-FITC (PharMingen), CD90-FITC (PharMingen), CD34-PE (PharMingen), CD14-FITC (Becton Dickinson), CD44-FITC (Becton Dickinson), CD105-PE (CALTAG, Burlingame, CA, USA), anti-FITC-alkaline phosphatase (LAP) antibody (Pharmigen), anti-bone sialoprotein (BSP) antibody (Calbiochem), and IgG-FITC and IgG-PE (Becton Dickinson). The employed LAP antibody clone has been reported to recognize human bone alkaline phosphatase (51,56). Bone sialoprotein is known as initiator of matrix mineralization and is upregulated during the mineralization phase of osteoblast differentiation (27).

cDNA Microarray

We employed a custom-made cDNA microarry chip that was constructed at the Institute for Molecular Biology and Tumor Research Marburg as described previously (8). There were 9600 independent cDNA clones selected from a cDNA library obtained from Research Genetics (Invitrogen Corporation, Carlsbad, CA, USA). RNA was amplified in vitro according to modified "antisense" method (5,66). cDNA synthesis from generated antisense RNA was performed using indirect labeling with CyScribe cDNA post labeling kit (Amersham Pharmacia Biotec, Buckinghamshire, England) according to the manufacturer's instructions in the presence of fluorescent Cy3 and Cy5 dyes. After purification and denaturation, the labeled targets were hybridized to the microarrays at 55°C overnight. After hybridization, the arrays were washed under stringent conditions to remove unspecific target binding and were subsequently air dried. Fluorescence signals were analyzed using an Affymetrix 418

Array™ Scanner with the Imagen® software Version 3.0. The expression level was deduced from the ratio of signal intensity (Cy5/Cy3).

Data Preprocessing and Analysis

For each spot, median signal and background intensities for both channels were obtained. To account for spot differences, the background-corrected ratio of the two channels was calculated. Following the annotation of Yang et al. (92), we used the log $M = \log_2 R/G$ and the mean log intensity $A = \log_2\sqrt{RG}$, and the MA plots as described by Dudoit et al. (16). Here, R and G denote the measured fluorescence intensities after background subtraction for the probes labeled with the Cy5 and Cy3 dyes, respectively.

To balance the fluorescence intensities for the two dyes as well as to allow the comparison of expression levels across experiments, the raw data were standardized. First, we used an intensity-dependent standardization as described by Yang et al. (92) to correct for inherent and random bias on each chip. In a second step, a global standardization was applied to center the log ratios at zero. As each gene was spotted twice on the chip, and four arrays were analyzed, mean log ratios M were calculated for each gene. A cutoff of threefold expression in the mean log ratio was chosen to identify candidate genes for differentially expression.

Direct Sequencing Analysis

Spots with differentially regulated genes were corroborated using sequencing analysis. To this end, plasmids with cDNA inserts from Research Genetics library were first cultivated inside *E. coli* with LB medium and then DNA extracted using the QIAprep® Spin Miniprep kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions. Sequencing was performed with a DNA sequencing kit (Applied Biosystems, Warrington, UK) as described by the manufacturer on an ABI PRISM™ 377 DNA sequencing device (Applied Biosystems).

Real-Time PCR Analysis

For selected genes, a quantitative expression analysis was employed. RNA was extracted from MSC and skin fibroblasts using a commercial kit (RNeasy Qiagen) according to the manufacturer's instructions. From the same RNA previously used for preamplification and array hybridization, 1 μg RNA was taken for cDNA synthesis with the Omniscript™ RT-PCR kit from Qiagen as recommended by the manufacturer. β-Actin PCR was performed as control for the quality of cDNA on a thermal cycler PE 9600 (Applied Biosystems) as previously described (76), followed by visualization on an agarose gel. To quantify cDNA levels the QuantiTect™ SYBR® Green PCR kit (Qiagen) and 1 μl cDNA were used on an ABI PRISM 7700 Sequence Detector (Taqman™, Applied Biosystems) with the following amplification conditions: 45 cycles of three-step PCR; 94°C for 15 s, 56°C for 30 s, 72°C for 30 s for IGF2 and PPARGC1 primers and 94°C for 15 s, 58°C for 30 s, 72°C for 30 s for IGFBP5, LIM, and DKK3 primers after initial denaturation at 95°C for 15 min with QuantiTect™ SYBR® Green PCR kit (Qiagen) and 1μ 1 cDNA. Primer sequences are listed in Table 1. RNA levels were normalized using the β-actin housekeeping gene as described.

RESULTS

Human Bone Marrow Mesenchymal Cells Exhibit High Proliferation and Differentiation Abilities Compared to Skin Fibroblasts

To ensure that bone marrow-derived mesenchymal cells are true stem cells with capacity for self-renewal and differentiation, MSC were grown continuously up to 20–25 passages without loss of proliferation ability, viability, or differentiation capacity. Purity of MSC was confirmed by flow cytometric analysis: no hematopoietic cell, neither macrophages (CD14, CD45), blood progenitor cells (CD34, CD45), nor endothelial precursors (CD34, CD31) were detected (Fig. 1). Cloning efficiency of MSC as indicated by their ability to form CFU-F was checked in early and later cell passages and their potential to differentiate into osteocytes, chondrocytes, and adipocytes was tested in vitro. Skin fibroblasts were considered as mature counterparts and therefore also tested for their differentiation potential. These cells took considerably longer to grow confluent and could only be cultured for up to four passages. Therefore, we conclude that MSC but not skin fibroblasts resemble a stem cell phenotype with high proliferation capacity.

MSC exhibit a high level of leucocyte-alkaline phosphatase (LAP) and bone sialoprotein on flow cytometric analysis as well as increased extracellular calcium deposit (Kossa staining) after induction of osteogenic differentiation (Fig. 2A, B). However, no or only very light staining with LAP, bone sialoprotein, or extracellular calcium deposits was detected in fibroblasts derived from human skin (Fig. 2C). Thus, MSC, but not skin fibroblasts, exhibited the ability for ex vivo osteogenic differentiation. This was also true for chondrogenic differentiation.

Adipogenic differentiation capacity of MSC was demonstrated by increasing number of lipid vesicles,

TABLE 1
PRIMER SEQUENCES EMPLOYED IN REAL-TIME QUANTITATIVE PCR ANALYSIS OF FIVE SELECTED GENES THAT WERE FOUND TO BE REGULATED DIFFERENTLY IN MESENCHYMAL STEM CELLS COMPARED TO SKIN FIBROBLASTS

Accession Number	Gene Name	Primer Sequence	Product Size (bp)
N74623	Insulin-like growth factor 2	forward: 5′-TTTCCGCAGCTGT-GACCTG-3′ reverse: 5′-ATTGGAAGAACTTGCCCACG-3′	134
N89673	Peroxisome proliferative activated receptor γ	forward: 5′-TCAAATGAACAC GTGCACCC-3′ reverse: 5′-AAAGCACCAGTTCGGTTACCA-3′	140
T52830	Human insulin-like growth factor binding protein 5	forward: 5′-CAGTGCAAACCTTCCCGTG-3′ reverse: 5′-TGGCAGCTTCATCCCGTACT-3′	72
R92455	LIM protein	forward: 5′-TCCAGCAGGGAAACGAACTC-3′ reverse: 5′-GTTGAATTCT-TCTGGGTGCCA-3′	103
AA425947	Dickkopf homolog3	forward: 5′-TCTGGACCTCATCACCTGGG-3′ reverse: 5′-ACATACACCAGGCTGTGGCTG-3′	105

The accession number refers to the gen ID for GenBank research.

stained with Sudan black, but no fat vacuole was detected within the skin fibroblast culture. PCR analysis of the PPARγ2 gene, which is typically expressed in fat tissue, confirmed the adipogenic differentiation of MSC.

Therefore, we concluded that only MSC grown under standard conditions harbor stem cell plasticity, whereas skin fibroblast cannot be driven to differentiate into other mesenchymal lineages, indicating their mature state as skin stromal cells.

Mesenchymal Stem Cells Harbor Specific Gene Expression Signatures

Upon cDNA microarray analysis differential gene expression levels in MSC compared to mature fibroblasts was detected in about 80 from 9600 analyzed genes. Only genes with more than threefold difference in the expression level were considered for analysis.

Our data show that typical connective tissue genes encoding proteins like matrilin or extracellular matrix protein-1 (33,54) often had lower copy numbers in MSC versus skin fibroblast (Table 2), confirming the fibroblast progenitor status of MSC. In concordance with this finding, genes that occur in mature mesoderm-derived tissues like bone, fat, smooth muscle, or endothelium were often found to be downregulated in MSC [i.e., *AE-binding protein-1* (74), the *complement-regulatory gene decay-accelerating factor (DAF)* (50), *fatty acid binding protein 3*, and *PPARγ coactivator* 1 (18,38,71)] (Table 2).

Surprisingly, several genes of ectoderm origin, like *contactin* and *G-protein-coupled receptor*, which are considered as specific genes for neural tissues (10, 75), were downregulated in MSC. In addition we found that *cadherin 2* (46,47), *scrapie responsive protein 1* (15), and *netrin* (2,35,93), which are all detected in developmental neural tissue, were expressed in high amounts in MSC. Nevertheless, *neuritin* (57) and *contactin* (62,63), which seem to be involved in neural development as well, were downregulated in MSC.

Interestingly, several other genes that are somehow involved in embryogenesis and organ development were found to be regulated differently in MSC, corroborating the progenitor cell character of MSC for tissues of all dermal layers. All these embryonic and developmental genes are summarized in Table 3.

We also detected about 30 ESTs (expressed sequenced tags) and some other genes like dickkopf-3 (Dkk-3/REIC), a tumor suppressor gene whose promoter is often methylated in human tumor cells (37,61), that was found to be downregulated in MSC (Table 2).

Direct Sequencing and Quantitative PCR Analysis Revealed Correct Gene Identity and Expression Quantification in MSC

Out of the 80 differently expressed genes 45 clones were selected randomly and their identity was confirmed by direct sequencing (Table 2). From the 45 sequenced genes one was a mixed clone and another one revealed to be another gene (i.e., it did not match the array spot). Therefore, the quality of our custom-made array chip proved to be valid and reliable regarding the identified genes.

In addition, real-time quantitative PCR analysis revealed a good correlation to the quantity of gene expression measured by our gene array system. We performed quantitative PCR analysis for five representative genes and demonstrated an equal expression level of the identified genes (Table 4). Interestingly, the level of up- or downregulation seemed to be even

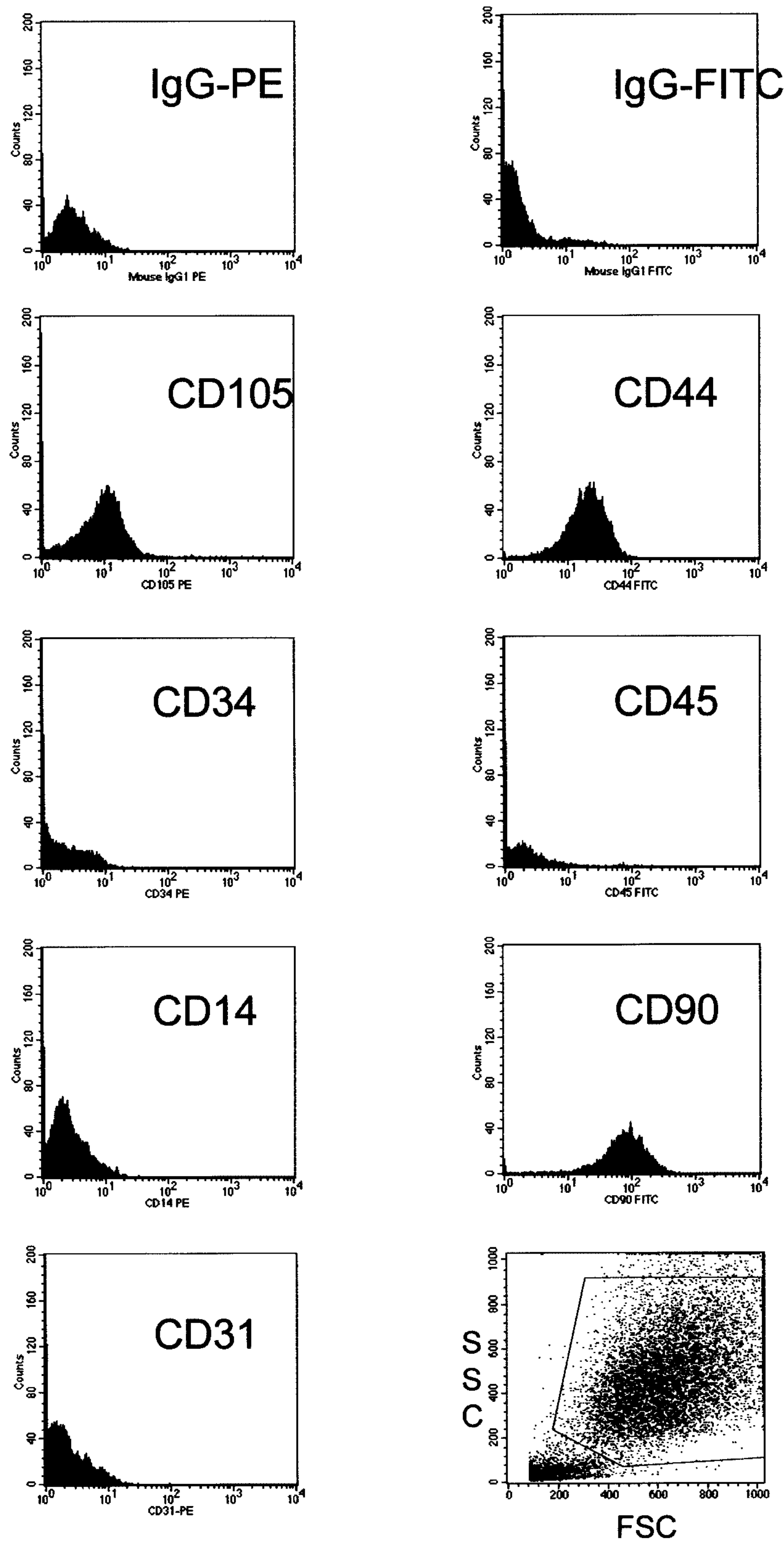

Figure 1. Homogeneous antigen profile of mesenchymal stem cells. Mesenchymal stem cells show homogeneous surface expression of CD44, CD90, and CD105. Staining for hematopoietic (CD45, CD14, CD34) and endothelial (CD34, CD31) cells was negative, thus proving purity and homogeneity of mesenchymal stem cell cultures.

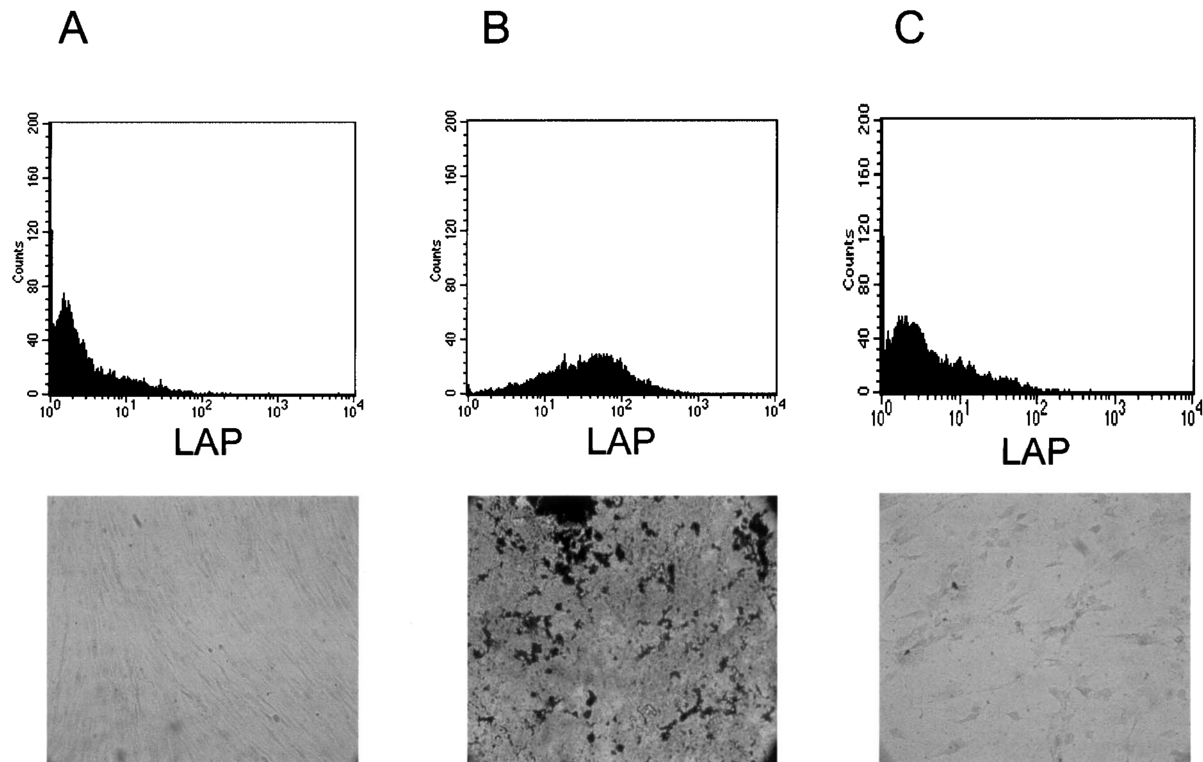

Figure 2. Mesenchymal stem cells but not skin fibroblasts harbor osteogenic differentiation abilities. Mesenchymal stem cells without induction of osteogenesis show no expression of alkaline phosphatase (LAP) by FACS analysis or calcium deposits in von Kossa staining (A). After induction of bone differentiation elevated LAP levels and a significant amount of calcium deposits can be detected in former mesenchymal stem cells (B), but not in skin fibroblasts (C).

more pronounced employing real-time quantitative PCR analysis. Thus, our array data were confirmed and proved to be specific and sensitive without overestimation of expression differences.

DISCUSSION

Although human bone marrow mesenchymal stem cells have a similar morphology and resemble the same immunophenotype such as human skin fibroblasts, distinct biological differences can be found with regard to growth pattern and differentiation capabilities. In order to address whether this is paralleled by different gene expression profiles we hybridized mature and immature fibroblastic cell types against each other. Therefore, in our approach genes and signaling pathways conferring stem cell abilities were likely to be revealed in MSC rather than typical fibroblastic lineage genes, like genes encoding for vimentin, different collagens, stromal cell-derived factor 1, or fibronectin. In concordance with this we found that those genes encoding for structural proteins typically active in mature mesodermal tissues were expressed in lower amounts in MSC (i.e., *extracellular matrix protein 1*, *fatty acid binding protein 3*, peroxisome proliferative activated receptor γ coactivator 1, *AE-binding protein 1*, or *matrilin 2*).

Interestingly, we discovered several differently regulated genes that play a role in neuroectodermal development: *G-protein-coupled receptor* and *contactin*, genes found in mature neural tissue (10,75), were downregulated in MSC. In contrast *netrin*, *cadherin 2*, and *scrapie responsive protein 1*, which can typically be found in developing neural tissue, were found to be upregulated in MSC. From these data we conclude a neuronal precursor status of MSC, which is confirmed by the finding of human adult MSC differentiating along neuronal pathway in vitro (32,36,42,70). In concordance with other groups, who found nestin and neurofilament H expressed in MSC (84), all these data confirm the ubiquitous germline, ectodermal, endodermal, and mesodermal gene expression profile of MSC (89).

Beyond that, we were able to demonstrate that MSC regulate a variety of genes that are abundantly expressed in the early embryo or during organ development, although MSC are considered to be “adult” stem cells. Most of these developmental genes were found to be upregulated, such as *distal-less homeo box 5 gene*, which plays a role in embryonic organ

TABLE 2
GENES THAT WERE FOUND TO BE UPREGULATED AND DOWNREGULATED IN MSC VERSUS SKIN FIBROBLASTS

Expression Level	Accession Number	Gene Name	Function (References)
Upregulated genes			
10.0	AA430540	Collagen, type IV, alpha 2	extracellular matrix protein
8.8	N74623	Insulin-like growth factor-2	regulation of normal cell growth (59)
7.2	AA136707	Procollagene-lysine, 2-oxoglutarate 5-dioxygenase	collagen synthesis
6.0	AA459308	Elastin	protein that provides the property of elastic recoil in dermis, lungs, and blood vessels (67)
5.6	N74882	Distal-less homeobox 5	organ development, limb initiation (21,95)
5.4	AA460975	Scrapie responsive protein 1	expressed by cells of neural origin in mouse embryo (15)
4.6	AA482119	Inhibitor of DNA binding 3	plays a role in keeping precursor cells immature (94)
4.5	T52830	Human insulin-like growth factor binding protein 5	growth and differentiation (26,53)
4.4	AA421819	Cadherin 6, type 2, K-cadherin	cell adhesion molecule, expressed in normal kidney and renal cell carcinoma (65)
4.0	W49619	Cadherin 2, type 1, N-cadherin	neural development (46,47)
4.0	AA487193	Secreted frizzled-related protein 4	transmembrane receptor; WNT network (19,34)
4.0	W72803	EGF-TM7 latrophilin-related protein	transmembrane receptor, neutrophil migration (41)
3.7	R76614	Netrin 4	neuronal development (2,35,93)
3.7	AA074535	Hematopoetic PBX-interacting protein	organization of cytoskeleton (1)
3.4	R92455	LIM protein	angiogenesis and hematopoiesis (90,91)
3.4	AA402754	Eyes absent (*Drosophila*) homolog 2	dynamic expression during development (17)
3.4	H79023	Disintegrin and metalloproteinase (meltrin α) ADAM 12	disintegrin and metalloproteinase is expressed in human and rat brain, developing and regenerating heart, and skeletal muscle (7)
3.3	R55185	EST, highly similar to IRX3 mouse II	IRX3 is involved in WNT signaling in developing neural tissue (9) and developing heart (12)
3.3	AA417279	Protein tyrosine phosphatase, non-receptor type substrate 1	expressed in rat kidney (55)
3.1	N79778	Extracellular matrix protein 2	extracellular matrix protein, female organ and adipocyte specific
3.1	R82176	MAD (mothers against dpp = decapentaplegic) homolog 7	embryonic midgut development in drosophila; BMP-4 signaling pathway (58)
Downregulated genes			
−30.3	N32768	Pregnancy specific beta-1-glycoprotein 3	immunomodulating function (80)
−25.0	W48852	Cysteine knot superfamily 1	growth factors (52)
−10.6	R48303	Dermatopontin	proteoglycan-binding cell adhesion protein, interacts with TGF-β (64)
−10.5	AA496334	Dynamin 1	endocytosis (11)
−10.0	AA430540	Collagen, type IV, alpha 2	extracellular matrix protein
−9.3	AA148548	Fatty acid binding protein 3	fatty acid metabolism, trafficking, and signaling (71)
−8.7	AA644088	Cathepsin C	activates serin proteases like progranzymes (83,88)
−8.1	N50845	Contactin 3	adhesion molecule, neural development (62,63)
−7.0	H09748	B-cell CLL/lymphoma 11B	zinc finger protein
−6.8	T50121	Kreisler maf-related leucine zipper homolog	transcription factor, activated HOXb-3 in segmental regulation (48)
−6.8	W49781	Leupaxin	phosphotyrosine protein most homologous to paxillin preferentially expressed in hematopoietic cells (45)
−6.3	R66101	Neuritin	neurogenesis (57)
−5.8	W35153	G protein-coupled receptor, family C, group 5, member B	
−5.7	AA425947	Dickkopf (*Xenopus laevis*) homolog 3	candidate tumor suppressor gene (37,61)
−4.0	N89673	Peroxisome proliferative activated receptor γ coactivator 1	fat metabolism, transcriptional coactivator (18,38)
−3.8	AA490462	AE-binding protein 1	regulated in adipogenesis and vascular smooth muscle cell differentiation (74)
−3.6	AA071473	Matrilin 2	development of cartilage and bones (23)
−3.6	R09561	Decay accelerating factor for complement	membrane-bound complement-regulatory protein (CD55, Cromer blood group system) (81)
−3.5	N79484	Extracellular matrix protein 1	bone development and angiogenesis (33,54)
−3.4	N23996	SWAP-70 protein	Signaling of membrane ruffling (78)
−3.3	N98485	Forkhead box F2	Potential transcriptional factor
−3.2	H19315	Contactin 1	neural cell adhesion molecule

TABLE 3
MESENCHYMAL STEM CELLS DISPLAY A DIFFERENTIAL EXPRESSION OF GENES INVOLVED IN EMBRYOGENESIS AND TISSUE DEVELOPMENT

Upregulated in MSC	Downregulated in MSC
Insulin-like growth factor II	Neuritin
Netrin	Contactin 3
LIM protein	Kreisler (Kmrl1)
Secreted Frizzled-related protein 4	Extracellular matrix protein 1
Inhibitor of DNA binding 3	
Distal-less homeo box 5	
Scrapie responsive protein 1	
Human insulin-like growth factor BP5	
Cadherin 2, type 1	
Eyes absent homolog 2	
MAD homolog 7	

development (95) and especially limb initiation (21), but a few of them were downregulated as well, as depicted in Table 3. Pronounced expression of developmental genes in MSC was corroborated by another group comparing gene expression of MSC versus CD34 (79) and indicates inherent plasticity and potential remodeling facilities of MSC.

Although results of gene array printings vary considerably because of different stem cell sources, stem cell purity (87), different array chips probed, or statistical analysis employed in data interpretation (20,22), identification of common "stem cell genes" in two or more studies is statistically significant and warrants further investigation. In our approach we identified five genes that were already described as "stem cell" genes before by comparing the gene expression profile of hematopoietic, neural, and embryonic cells by microarray analysis (30,72).

Among the proteins encoded by these enhanced genes were *four and a half LIM protein*, which plays a pivotal role in yolk sac erythropoiesis and in the proper development of all hematopoietic lineages in the adult individual (91) as well as angiogenesis (90) and interacts with *human insulin-like growth factor binding protein 5* (IGFBP-5) (3) (Table 3). IGFBP-5 is the most conserved IGFBP across species among the family of IGF binding proteins (IGFBP-1 to -6), and was identified as an essential regulator of physiological processes in bone, kidney, and mammary gland (6,14,49,77,86). In addition, IGFBPs appear to play a decisive role in control of proliferation of specific tumor cell lines (44,82) as well as limb development (53) and growth of smooth muscle cells (39).

The *distal-less homeo box 5 gene* was also described as a "stem cell" gene before (30) and was found to be enriched in our MSC. Its upregulation seems to be a unique stem cell feature, because it is found in four different stem cell entities.

In addition we identified two MSC "stemness" genes of the Wnt signaling network, which is involved in embryo development and cancer formation [i.e., the gene for *secreted frizzled-related protein 4*, a protein abundantly expressed in the early embryo (30,34) and *dickkopf-3* (*dkk-3*), related to Dickkopf protein 1 (dkk1) (34)]. *dickkopf-3* also resembles a candidate tumor suppressor gene responsible for aging processes (37,61,85). In summary, these regulators of this Wnt/β-catenin pathway seem to play a prominent role in MSC, because they have been described as an autocrine signaling mechanism operating in MSC by different investigators (19) also.

Although the precise signaling pathways that determine the differentiated fate of MSC is not fully understood yet, our data confirm the idea that, unlike skin fibroblasts, adult bone marrow mesenchymal stem cells in humans may have true stem cell characteristics. Our finding is in concordance with data from other groups, who approached the gene expression profiling in mesenchymal stem cells upon in vitro differentiation into osteogenic, chondrogenic, and adipogenic tissue (4,13,28,29,40,60), but only one to three overlapping genes are confirmed in each gene set employed. The issue of reproducibility of gene expression profiles under different conditions (culture passages, media and serum conditions, array slides) has been discussed extensively (20,22,40,87).

In conclusion, no single study can confidently identify bona fide stem cell genes; cross-validation of gene array results generated independently by different investigators remains to be crucial. With our approach we were able to confirm gene expression profiles that were described for stem cells and developmental processes before, and future studies may reveal more details in signaling pathways responsible for the respective differentiation processes.

TABLE 4
VERIFICATION OF GENE ARRAY RESULTS BY REAL-TIME QUANTITATIVE PCR ANALYSIS

Gene (Accession No.)	Relative Gene Expression	
	cDNA Array	Real-Time PCR
Peroxisome proliferative activated receptor γ (N89673)	−4.0	−6.6
Dickkopf homolog 3 (AA425947)	−5.7	−5.4
LIM protein (R92455)	3.4	12.2
Insulin-like growth factor-2 (N74623)	8.8	91.7
Human insulin-like growth factor binding protein 5 (T52830)	4.5	10.3

Gene expression measured with both techniques shows good correlation. Differences in expression levels seem to be more pronounced with real-time PCR analysis.

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