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Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood

Wolfgang Wagner[a], Frederik Wein[a], Anja Seckinger[a], Maria Frankhauser[b], Ute Wirkner[c], Ulf Krause[a], Jonathon Blake[c], Christian Schwager[c], Volker Eckstein[a], Wilhelm Ansorge[c], and Anthony D. Ho[a]

[a]*Department of Medicine V, University of Heidelberg, Heidelberg, Germany;* [b]*Cytonet GmbH, Heidelberg, Germany;* [c]*Biochemical Instrumentation Program, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany*

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Objective. **Various preparative protocols have been proposed for the acquisition and cultivation of mesenchymal stem cells (MSC). Whereas surface antigen markers have failed to precisely define this population, microarray analysis might provide a better tool for characterization of MSC.**

Methods. **In this study, we have analyzed global gene expression profiles of human MSC isolated from adipose tissue (AT), from umbilical cord blood (CB), and from bone marrow (BM) under two growth conditions and have compared them to terminally differentiated human fibroblasts (HS68). Profiles were compared using our Human Genome Microarray representing 51.144 different cDNA clones.**

Results. **Cultured with the appropriate conditions, osteogenic and adipogenic differentiation could be confirmed in all MSC preparations but not in fibroblasts. No phenotypic differences were observed by flow cytometry using a panel of 22 surface antigen markers. Whereas MSC derived from different donors using the same culture procedure yielded a consistent and reproducible gene expression profile, many genes were differentially expressed in MSC from different ontogenetic sources or from different culture conditions. Twenty-five genes were overlapping and upregulated in all MSC preparations from AT, CB, and BM as compared to HS68 fibroblasts. These genes included *fibronectin*, *ECM2*, *glypican-4*, *ID1*, *NF1B*, *HOXA5*, and *HOXB6*. Many genes upregulated in MSC are involved in extracellular matrix, morphogenesis, and development, whereas several inhibitors of the Wnt pathway *(DKK1*, *DKK3*, *SFRP1)* were highly expressed in fibroblasts.**

Conclusion. **Our results have provided a foundation for a more reproducible and reliable quality control using genotypic analysis for defining MSC.** © **2005 International Society for Experimental Hematology. Published by Elsevier Inc.**

Introduction

Mesenchymal stem cells (MSC) represent an archetype of multipotent somatic stem cells that hold promise for application in regenerative medicine. Given the appropriate microenvironment, MSC could differentiate into cardiomyocytes or even cells of nonmesodermal derivation including hepatocytes and neurons [1–5]. Although mesenchymal stem cells were originally isolated from bone marrow [6,7], similar populations have been reported in other tissues. Human MSC have been isolated from adipose tissue [8], umbilical cord blood [9–12], peripheral blood [13,14], connective tissues of the dermis, and skeletal muscle [15]. Applying modifications in culture conditions, the group of Catherine Verfaillie reported on a special subset of MSC population which they named multipotent adult progenitor cells (MAPC). MAPC have been shown to produce cells with characteristics of visceral mesoderm, neuroectoderm, or endoderm. When injected into an early blastocyst a single MAPC contributed to the development of various tissues

Offprint requests to: Anthony D. Ho, M.D., Department of Medicine V, University of Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany; E-mail: anthony_dick.ho@urz.uni-heidelberg.de

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[3]. Recently, Kogler et al. have described another subset of MSC derived from human cord blood (CB) which they called "unrestricted somatic stem cells" (USSC). These cells were able to differentiate into many cell types including hepatic cells and cardiomyocytes [12]. All these experiments demonstrated that variations in conditions have a significant impact on the developmental potential of the populations generated, albeit the initial cell material could be phenotypically identical.

MSC have been defined by their plastic adherent growth and subsequent expansion under specific culture conditions, by a panel of nonspecific surface antigens and by their in vitro and in vivo differentiation potential [16]. Induction of differentiation into osteoblasts, adipocytes, and chondrocytes under appropriate culture conditions has been demonstrated extensively [7,12,17–22]. In contrast, human fibroblasts do not possess this in vitro differentiation capacity [7]. Phenotypically MSC have been defined as $CD29^+$, $CD44^+$, $CD90^+$, $CD105^+$, and negative for hematopoietic lineage markers and HLA-DR [16,23–26].

Due to their accessibility, expandability, and multipotentiality, MSC hold promise for clinical applications [27–37]. However, the lack of common standards and of a precise definition of initial cell preparations remains a major obstacle for research and application of MSC. The heterogeneity of the starting population for most of the trans-differentiation experiments renders comparison of results between different groups difficult and might also partly account for the lack of reproducibility of some of the initial reports. The significance of establishing standards and guidelines for clinical applications can best be demonstrated by the evolvement of bone marrow or blood stem cell transplantation from a highly experimental procedure to standard therapy strategy for several malignant and hereditary diseases [38]. Performed anecdotally in patients with incurable diseases such as hereditary immunodeficiencies or acute leukemias in the mid-1960s, blood stem cell transplantation now offers chances of durable cure. Standards and guidelines have been developed during the early 1990s and these have laid the foundation for the present international standard operating procedures (SOP) for blood stem cell transplantation. A precise characterization of MSC intended for human use thus represents a *conditio sine qua non* for future development and for exploitation of stem cell research for clinical application.

In this study we have isolated MSC from bone marrow, adipose tissue, and umbilical cord blood and have compared their genome-wide expression profiles with nonmultipotent fibroblasts. The aim of this study was to analyze reproducibility of generation of MSC under standardized conditions, to compare molecular genetic make-up of MSC derived from different ontogenic sources, and to identify genes that are commonly upregulated in all preparations of MSC which might serve as a novel parameter for the definition of MSC.

Material and methods

Cells and cell culture

Mesenchymal stem cells from bone marrow. MSC from the bone marrow (BM) were isolated form the same donors under two different growth conditions (M1 and M2). Bone marrow aspirates were obtained from the iliac crest of four healthy donors aged 25–35 years after approval by the Heidelberg University Ethical Board (approval nos. 042/2000 and 251/2002). About 10 to 30 mL bone marrow aspirate were collected in a syringe containing 10,000 IU heparin to prevent coagulation. The mononuclear cell fraction was isolated by Biocoll density gradient centrifugation ($d = 1.077$ g/cm^3; Biochrom, Berlin, Germany).

BM-MSC-M1 were cultivated as described by M. Reyes and colleagues before [21]. In brief, mononuclear cells were plated in expansion medium (M1) at a density of 10^5 cells/cm^2 in tissue culture flasks (Nunc, Wiesbaden, Germany) coated with 10 ng/mL fibronectin (Sigma, Deisenhofen, Germany). The expansion medium consists of 58% Dulbecco's Modified Eagle's Medium—Low Glucose (DMEM-LG, Cambrex, Apen, Germany) and 40% MCDB201 (Sigma), 2% fetal calf serum (FCS; StemCell Technologies, Vancouver, BC, Canada), supplemented with 2 mM L-glutamine, 100 U/mL Pen/Strep (Gibco, Eggenstein, Germany), 1% insulin transferrin selenium, 1% linoleic acid bovine serum albumin, 10 nM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate (all from Sigma), platelet-derived growth factor, and epidermal growth factor (10 ng/mL each, R&D Systems, Wiesbaden, Germany). On reaching 80% confluency, cells were trypsinized with 0.25% trypsin / 1 mM EDTA (Invitrogen, Karlsruhe, Germany) and replated at about 9000 cells/cm^2. Cells were expanded for 2 to 6 passages.

BM-MSC-M2 were cultivated in the commercially available Poietics Human Mesenchymal Stem Cell Medium (M2; PT-3001, Cambrex) following the manufacturer's instructions. About 10^5 cells/cm^2 were plated in tissue culture flasks without fibronectin coating. The plastic adherent cell fraction was reseeded at a density of about 9000 cells/cm^2. Cells were expanded for 2 to 6 passages.

Mesenchymal stem cells from adipose tissue. Human adipose tissue (AT) of four healthy donors aged 21 to 40 years was obtained from elective liposuction procedures under anesthesia after informed consent using guidelines approved by the Ethics Committee on the Use of Human Subjects (Cytonet, Heidelberg, Germany). AT-MSC-M1 were isolated as described before [22]. In brief, lipoaspirates were washed with sterile phosphate-buffered saline (PBS). A two-step digest in Krebs-Ringer (pH 7.4) buffered with 25 mM Hepes containing 20 mg/mL bovine serum albumin (BSA) and 1.5 mg/mL collagenase (CLS type I) was performed for 30 minutes at 37°C under permanent shaking, followed by filtration through a 250-μm mesh filter. Cell suspensions were centrifuged at 200*g* for 10 minutes, and contaminating erythrocytes were removed by erythrocyte lysis buffer pH 7.3. After washing, filtrate cells were cultivated in the same expansion medium M1 as described above for BM-MSC-M1 [21].

Mesenchymal stem cells from cord blood. Human umbilical cord blood (UCB) was collected after informed consent of the mother using the guidelines approved by the Ethics Committee on the use of Human Subjects by a standardized procedure using syringes

containing L-heparin as anticoagulant. After 2:1 dilution with PBS, mononuclear cells (MNC) were obtained by Ficoll density-gradient centrifugation (400*g* for 25 minutes). The cells were washed twice in PBS und seeded at a density of 1 to 3 $\times$ 10^6 cells/cm^2. Growth of adherent cells was initiated in myelocult medium (StemCell Technologies) with dexamethasone (10^{-7} M; Sigma-Aldrich), penicillin (100 U/mL; Gibco), streptomycin (0.1 mg/mL; Gibco), and glutamine (2 mM; Gibco) as previously described by Kogler et al. [12]. Nonadherent cells were removed after 72 hours, and the adherent cells were fed weekly with culture medium. Expansion of the cells was performed in Mesencult basal medium (M3; StemCell Technologies) with additive stimulatory supplements according to the manufacturer's instructions as described previously by L. Hou and colleagues [26].

Human fibroblasts. HS68 cells (human newborn foreskin fibroblasts) (ATCC; CRL-1635, Rockville, MD, USA) were cultured in DMEM-HG (Cambrex) with 2 mM L-glutamine, 100 U/mL Pen/Strep (Gibco), and 10% v/v fetal calf serum (FCS; StemCell Technologies). NHDF (normal human dermal fibroblasts) derived from foreskin (Promocell, Heidelberg, Germany) were cultured in M3.

In vitro differentiation

To induce osteogenic differentiation, cells were replated at 1 to 2 $\times$ 10^4 cells/cm^2 and cultured for three weeks in DMEM with 10% FCS (Invitrogen), 10 mM β-glycerophosphate, 10^{-7} M dexamethasone, and 0.2 mM ascorbic acid with media changes every 3 to 4 days as previously described [21,24]. After 21 days cells were analyzed by von Kossa staining and alkaline phosphatase staining. To induce adipogenic differentiation, cells were plated at 1 to 2 $\times$ 10^4 cells/cm^2 and cultured for two weeks in 10% FCS, 0.5 mM isobutyl-methylxanthine (IBMX), 1 μM dexamethasone, 10 μM insulin, and 200 μM indomethacin, and Oil Red-O staining was performed after 21 days [7].

Immunophenotyping

MSC and HS68 were cultured in corresponding growth medium for 72 hours prior to analysis. Cells were labeled with the following anti-human antibodies: CD10-FITC, CD13-APC, CD14-FITC, CD29-FITC, CD34-PE, CD45-FITC, CD49d-PE, CD73-PE, CD90-APC, CD106-PE, CD117-PE, CD166-PE, SSEA-4-FITC, HLA-ABC-PE, CD44-PE (all Becton Dickinson [BD], San Jose, CA, USA); CD24-FITC (Dako, Hamburg, Germany); CD31-FITC (Caltag, Burlingame, CA, USA); CD36-FITC, CD38 FITC (Immunoctech, Marseille, France); CD105 PE (Serotech, Kidlington, UK); CD133 PE (Miltenyi, Bergisch Gladbach, Germany); HLA-DR FITC (Pharmingen, Hamburg, Germany). Mouse isotype antibodies served as respective controls (BD). More than 50,000 labeled cells were acquired and analyzed using a FACS-Vantage-SE flow cytometry system running CellQuest software (BD).

RNA isolation and probe synthesis

Cells were harvested upon reaching 80% confluency. About 2 $\times$ 10^6 cells from each fraction were lysed and total RNA isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). DNase treatment was performed (Qiagen). RNA quality was controlled with the RNA 6000 Pico LabChip kit (Agilent, Waldbronn, Germany). Linear amplification of 10 μg total RNA was performed by a one-round in vitro transcription using the Arcturus RiboAmp Kit (Acturus, Mountain View, CA, USA). RNA of HS68 cells was isolated four times and always amplified in parallel to the MSC samples. The amplified RNA was analyzed by the RNA Nano Lab Chip kit (Agilent, Waldbronn, Germany) and by the SpectraMAX plus photometer (Molecular Devices, Sunnyvale, CA, USA) at 260 nm. About 10 μg aRNA samples were then incubated with 3 μg Random Primer (Invitrogen) and labeled by amino-allyl coupling using the Atlas Glass Fluorescent Labeling Kit (Clontech, Palo Alto, CA, USA) and Cy3-/Cy5-monofunctional reactive dye (Amersham Biosciences, Little Chalfont, England).

The human genome microarray

For microarray analysis we used our Human Genome Microarray as described previously [39]. It represents the Unigene Set-RZPD3 composed of 51,145 cDNA clones, a very well characterized subset of the IMAGE cDNA clone collection (http://www.rzpd.de; http://image.llnl.gov/image). Further details about this microarray are provided under http://embl-h3r.embl.de and the techniques for hybridization and washing of the slides have been described in detail [39,40].

Statistical analysis

Gene expression profiles of four different types of MSC (BM-MSC-M1, BM-MSC-M2, AT-MSC-M1, CB-MSC-M3) were compared to HS68. Cells were analyzed from four independent donors for each culture condition and each source. Color-flip hybridizations were performed for every hybridization, resulting in a dataset of 32 co-hybridizations. Slides were scanned using the GenePix 4000B Microarray-Scanner (Axon Instruments, Union City, CA, USA) and analyzed by the ChipSkipper Microarray Data Evaluation Software (http://chipskipper.embl.de) as described before [39]. ESTs with a more than twofold mean ratio in signal intensity ($\log_2$ ratio > 1 or < -1) in the 8 corresponding hybridizations (including inverted ratios of color-flip hybridizations) were considered to be differentially expressed. False discovery rate (FDR) was estimated by simulations. Stochastic permutations of all experimental ratio values for each hybridization were used to create sets of virtual replications. A total of 10^5 simulations were performed and the average number of genes within the filter criteria was given as FDR [39]. Hierarchical clustering (Euclidian distance) was performed with TIGR MeV Ver.2.2 software (Institute of Genomic Research, Rockville, MD, USA) either using all spots with a high-quality signal in more than 28 of 32 co-hybridizations (12005 ESTs) or using a set of differentially expressed ESTs (4001 ESTs). All co-hybridizations were performed with the same reference RNA of HS68 cells and thus differential expression of MSC cultures could be determined by the difference of the two mean $\log_2$ ratio vs HS68. Student's t-test was used for the statistical analysis of $\log_2$ ratios and $p < 0.001$ was considered to be highly significant. Differentially expressed genes were further classified by GeneOntology terms using GoMiner software (http://discover.nci.nih.gov/gominer/) and representation in functional categories was analyzed by hypergeometric distribution ($p < 0.05$). The complete microarray data including the description of all spotted ESTs (according to Minimal Information About Microarray Experiments, MIAME requirements [41]) was submitted to the public microarray database ArrayExpress (http://www.ebi.ac.uk/arrayexpress/; accession number: E-EMBL-4).

RT-PCR analysis

Differential expressions observed by microarray analysis were verified by real time reverse transcriptase polymerase chain reaction (RT-PCR) with LightCycler technology (Roche, Mannheim, Germany) in 11 regulated genes and 3 housekeeping genes. Total RNA samples were reverse transcribed by Superscript II (Gibco) and semi-quantitative PCR was performed with the LightCycler Master SYBR Green kit (Roche) with 3 mM MgCl at 30 seconds preincubation at 95°C followed by 45 cycles of 5 seconds at 55°C, 15 seconds at 72°C, and 2 seconds at 95°C. PCR products were subjected to melting curve analysis and to conventional agarose gel electrophoresis to exclude synthesis of unspecific products. 18s rRNA primers were supplied by Ambion (Austin, TX, USA) and all other primers were synthesized by Biospring (Frankfurt, Germany). Primer sequences are provided in Table 1. The amplification efficiency of PCR products was determined by calculating the slope after semi-logarithmic plotting of the values against cycle number [39,42]. Differential expression was calculated in relation to ubiquitin B.

Results

Isolation of MSC from different tissues

Mesenchymal stem cells were isolated from human bone marrow, adipose tissue, and umbilical cord blood. BM-MSC-M1 and AT-MSC-M1 were isolated in the same culture medium (M1) with low serum content (2% FCS) as described by the group of Catherine Verfaillie for the isolation of MAPC [3,21]. In addition, BM-MSC-M2 were cultivated in a commercially available medium with 10% FCS from the same donor samples as for BM-MSC-M1 to determine the impact of culture conditions. CB-MSC could not be isolated under the growth conditions M1; thus we used the same culture medium as described by Kogler et al. [12] for the isolation of USSC to initiate cell culture and then switched to another expansion medium (CB-MSC-M3). MSC could be isolated from all of 35 bone marrow samples (100%) under both culture conditions (M1 and M2), from all of 9 AT samples (100%), and from 30 of 90 different CB samples (34%). All isolated MSC populations displayed a spindle-shaped morphology (Fig. 1). BM-MSC-M1 varied in cell size as compared to BM-MSC-M2 and cell divisions occurred preferentially in the smaller cells without intimate contact to surrounding cells. However, upon contact with surrounding cells BM-MSC-M1 became larger with long cell protrusions at both poles. AT-MSC-M1 displayed a homogeneous morphology while CB-MSC-M3 had a heterogeneous morphology ranging from elongated thin cells to flat round cells.

Multilineage capacity of MSC

MSC can be induced to differentiate along the adipogenic, osteogenic differentiation and chondrogenic lineages using specific culture media [12,21,22,43]. In all of our MSC populations osteogen differentiation could be induced as examined by von Kossa staining (Fig. 1) and alkaline phosphatase staining. Adipogenic differentiation could be confirmed following the standard protocols and analyzed by Oil Red-O staining [7]. Lipid vesicles could be observed in BM-MSC-M1, AT-MSC-M1, and BM-MSC-M2. Adipogenic differentiation was less obvious in CB-MSC-M3 (Fig. 1) [9]. In human NHDF fibroblasts and HS68 fibroblasts both osteogenic and adipogenic differentiation were not observed under these in vitro differentiation conditions [7].

Immunophenotypes of MSC and fibroblasts

A selection of surface markers was tested by flow cytometric analysis (Fig. 2). All types of MSC and fibroblasts were negative for CD10, CD14, CD24, CD31, CD34, CD36, CD38, CD45, CD49d, CD117, CD133, SSEA4, and HLA-DR while they were positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166, and HLA-ABC as previously described by other authors [10,12,21,22]. Fluorescence cytometry could not discern any distinct characteristics exhibited by the MSC populations derived from different tissues. In comparison to HS68 fibroblasts, no obvious difference in the expressions of these surface antigens could be observed. Thus, this panel of surface markers does

Table 1. Primer Pairs designed for LightCycler RT-PCR

Target	Forward-primer	Reverse-primer
18s rRNA	5′-TCAAGAACGAAAGTCGGAGG-3′	5′-GGACATCTAAGGGCATCACA-3′
GAPDH	5′-ATGGCACCGTCAAGGCTGAGA-3′	5′-GGCATGGACTGTGGTCATGAG-3′
FN1	5′-TGCTTAGGCTTTGGAAGTGG-3′	5′-TGCCACTGTTCTCCTACGTG-3′
EDG3	5′-AGTACTGGATTAAGAAAACAACAACA-3′	5′-TGGCTCTCTGATGCATTTTG-3′
URB	5′-GCAGAGGAAACTGGCTGAGT-3′	5′-GCAACTTCGGAGACAGGAAA-3′
MEOX2	5′-CTGCATGAATACATCACATATGAAAA-3′	5′-GGCAAATCAGTTACCTTGCAG-3′
CD36	5′-TTTGTTCTTCCATCCAAGGC-3′	5′-TCCAATATCCCAAGTATGTCCC-3′
TBX5	5′-TCGCATAGGGACACTCACTT-3′	5′-GAAACCCAGCATAGGAGCTG-3′
CES1	5′-AAGGCAACTGGACCAGAAGA -3′	5′-CCACAATCACAGATGGGACA-3′
NCAM1	5′-TTGTTGCATTTTGGGTTCAA-3′	5′-CATGTGAATCAGTGCGGTCT-3′
TWIST2	5′-CAGCCACACTGCAGTCACTT-3′	5′-ACGCCGCTATTCTTTTCCTT-3′
DKK1	5′-CATTTGTCATTCCAAGAGATCC-3′	5′-TAAAGGTGCTGCACTGCCTA-3′
ITGA1	5′-CAGCTCTGCAAGTGAATACCA-3′	5′-CAATCCCAGTTGGGTACAGC-3′
Ubiquitin	5′-ATCACCCTTGGAGGTGGAG-3′	5′-GAAAGAGTACGGCCATCTTCC-3′

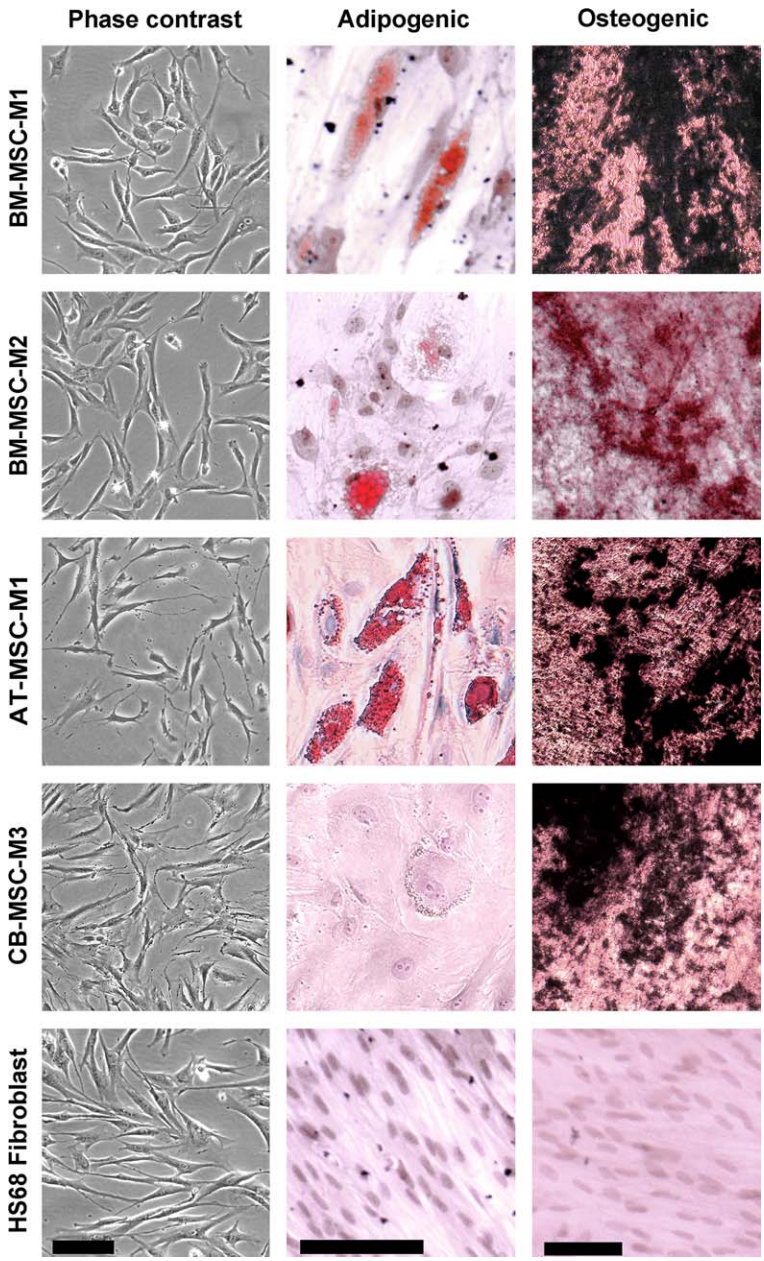

Figure 1. Morphologic comparison of MSC isolated under different conditions. Mesenchymal stem cells were isolated from the bone marrow under two different culture conditions (BM-MSC-M1 and BM-MSC-M2), from adipose tissue (AT-MSC-M1) and from umbilical cord blood (CB-MSC-M3). All cells were plastic adherent with a spindle-shaped morphology. AT-MSC-M1 appeared to be smaller while CB-MSC-M3 had a heterogeneous morphology ranging from flat round to thin elongated appearance. In all of our MSC populations adipogenic and osteogenic differentiation could be induced as examined by Oil Red-O staining or von Kossa staining. Adipogenic differentiation was less obvious in CB-MSC-M3. Adipogenic and osteogenic differentiation was not observed under the same differentiation conditions in human fibroblasts (HS68). Scale bar is equivalent to 100 μm.

not present itself as sufficient for the identification or definition of MSC.

Gene expression profiles of MSC

Genome-wide expression profiles of MSC were analyzed using our Human Transcriptome Microarray representing 51,144 different cDNA clones of the UniGene set RZPD3 [39]. Samples of BM-MSC-M1, BM-MSC-M2, AT-MSC-M1, or CB-MSC-M3 were co-hybridized with human HS68 fibroblasts. For each source of MSC, four individual donors were analyzed and a color-flip hybridization was performed for every hybridization. A total of 4001 different ESTs were differentially expressed in at least one type of MSC as compared to HS68 fibroblasts (Fig. 3A). This set of genes was used for hierarchical cluster analysis to determine reproducibility of culture-isolation conditions as well

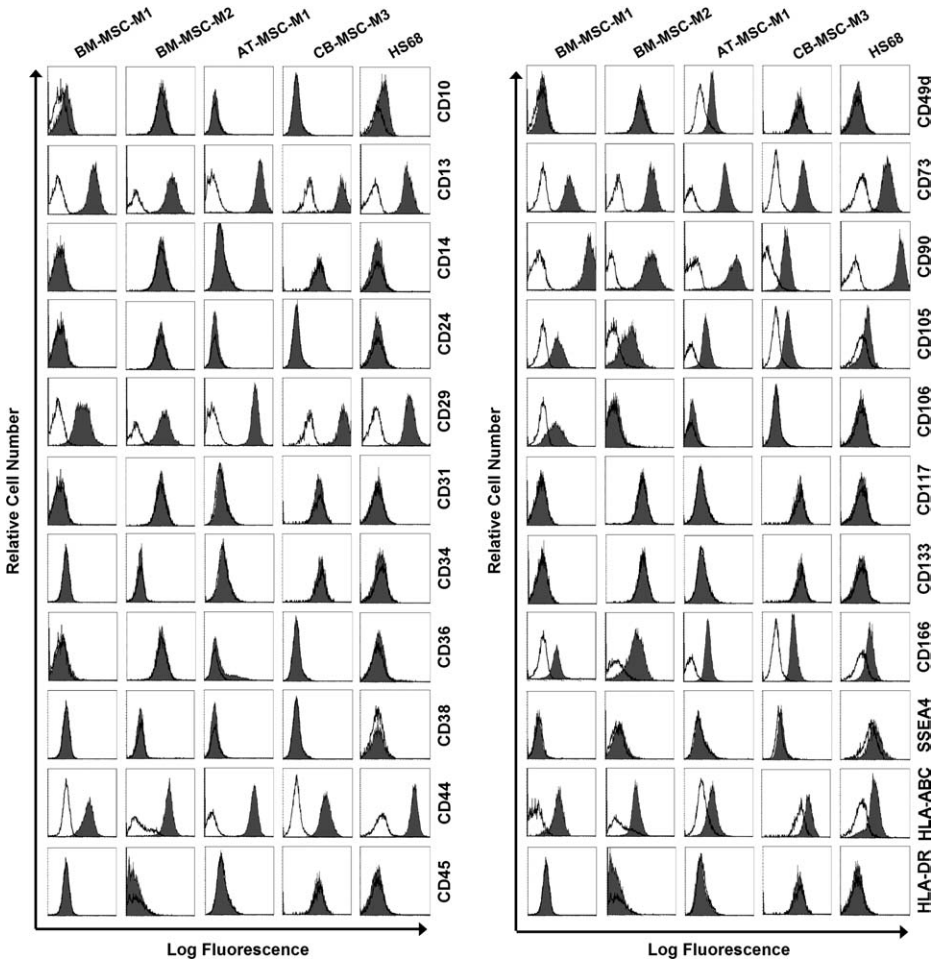

Figure 2. Immune phenotype of MSC. BM-MSC-M1, BM-MSC-M2, AT-MSC-M1, CB-MSC-M3, and HS68 cells were labeled with antibodies against the indicated antigens, and analyzed by flow cytometry. Representative histograms are demonstrated (gray). The respective isotype control is shown as black line. The staining pattern of MSC preparations and of HS68 fibroblasts was highly similar and thus these markers are not sufficient for definition of MSC.

as the relationship of the four different preparations of MSC. Similar results were obtained by unsupervised cluster analysis using all spots on the microarray with consistent signal intensity (results not shown). The dendrogram in Figure 3C demonstrates the close correlation of the color-flip experiments (technical replicas). MSC isolated under the same culture conditions clustered together while MSC from different tissues or culture media could be found in different clusters.

Forty-seven different ESTs (FDR = 0) including 25 characterized genes were more than twofold higher expressed in all preparations of MSC vs HS68 cells (Table 2). Among these were several genes that contribute to the formation of extracellular matrix including fibronectin 1 (FN1), chondroitin sulfate proteinglycan 2 (CSPG2), extracellular matrix protein 2 (ECM2), latent TGF β binding protein 1 (LTBP1), and glypican 4 (GPC4). Several transcription factors were upregulated in all preparations of MSC. These included the delta sleep inducing peptide (DSIPI), inhibitor of DNA binding 1 (ID1), and nuclear factor I/B (NFIB) as well as homeobox genes A5 and B6 (HOXA5 and HOXB6; in CB-MSC-M1 only 1.7-fold and 1.9-fold higher expressed). In contrast, a more than twofold lower expression in each of the four different MSC preparations was found in 206 different ESTs (FDR = 0). These included 30 characterized genes that were more than fourfold downregulated (Table 3). Among these were several proteins that modulate or cleave collagen and other extracellular matrix proteins like lysyl oxidase-like 4 (LOXL4) and matrix metalloproteinases 1, 3, 10 (MMP1,3,10). Several genes that were upregulated in fibroblasts have been associated with neuronal differentiation. They were nestin (NES); slit homolog 2 (SLIT2), which functions in axon guidance; and stathmin-like 2 (STMN2), which is probably involved in transcriptional regulation of neural differentiation. Neural cell adhesion molecule 1 (NCAM1) and integrin, α 2 (ITGA2) represent adhesion proteins. T-box5 (TBX5), involved in mesodermal regulation, as well as Twist homolog 2 (Twist2), which may inhibit osteoblast maturation, were also less expressed in MSC.

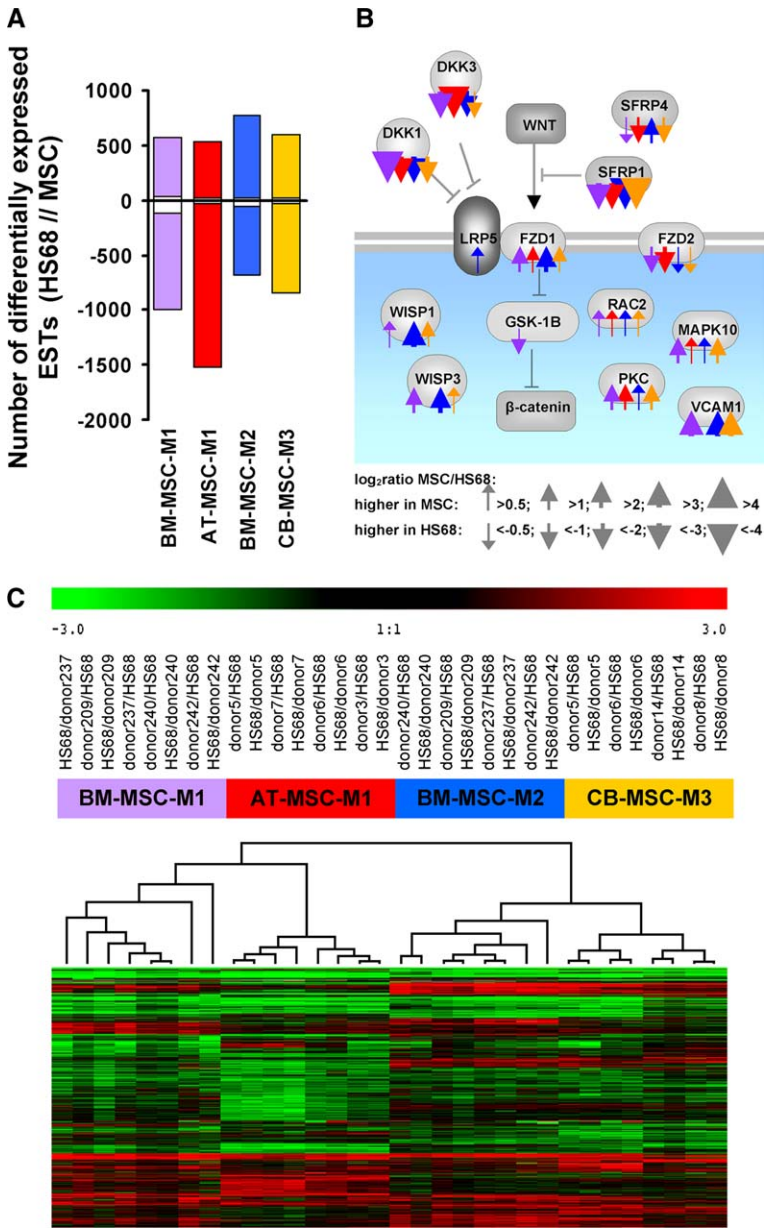

Figure 3. Analysis of differential gene expression. For each of type of MSC (BM-MSC-M1, AT-MSC-M1, BM-MSC-M2, and CB-MSC-M3) the number of ESTs that revealed a more than twofold mean differential expression vs human fibroblasts (HS68) in the eight corresponding co-hybridizations is presented **(A)**. In total, 4001 different ESTs passed these filter criteria and false discovery rate (FDR) is presented in white columns. Several genes differentially expressed between different MSC and fibroblasts (HS68) are involved in the Wnt pathway (B). Ratio of differential expression is indicated by arrows. Arrow up: higher expressed in MSC; arrow down: higher expressed in HS68. Arrow size correlates with $\log_2$ ratio as indicated and SD in eight corresponding co-hybridizations was less than the mean $\log_2$ ratio. The 4001 differentially expressed ESTs were further analyzed by hierarchical clustering **(C)**. Red: upregulated in MSC; green: upregulated in HS68. The dendrogram visualizes the relationship of different MSC preparations.

Several genes involved in the Wnt pathway are differentially expressed in MSC in comparison to HS68. Figure 3B demonstrates that inhibitors of the Wnt pathway including dickkopf homolog 1 and 3 (DKK1, 3), secreted frizzled-related protein 1 and 4 (SFRP1, 4), and frizzled 2 were consistently higher expressed in HS68. In contrast, frizzled 1 and several downstream activators of this pathway (WISP1, WISP3, RAC2, MAPK10, VCAM1, and PKC) were higher expressed in MSC.

Functional classification of differentially regulated genes

Differentially regulated genes were classified by functional annotation using GeneOntology terms. Here, genes were considered as differentially expressed if mean $\log_2$ ratio was higher than standard deviation (SD) in 32 co-hybridizations of the different types of MSC preparations with HS68 fibroblasts. A total of 160 different genes were higher expressed in MSC and were functionally classified while 418 classified genes were higher expressed in

Table 2. Genes upregulated (>twofold) in all sources of MSC vs human fibroblasts (HS68)

			BM-MSC-M1		AT-MSC-M1		BM-MSC-M2		CB-MSC-M3	
Gene name	Shortcut	Acc.No.	Log_2ratio±*SD*		Log_2ratio±*SD*		Log_2ratio±*SD*		Log_2ratio±*SD*	
Extracellular matrix										
extracellular matrix protein 2	ECM2	n79778	**2.19**	*1.00*	**1.59**	*0.80*	**2.80**	*1.02*	**1.68**	*2.21**
extracellular matrix protein 2	ECM2	ai886645	**2.17**	*1.52*	**1.23**	*0.99*	**3.14**	*1.12*	**1.79**	*2.39**
fibronectin 1	FN1	w57892	**2.72**	*0.38*	**2.38**	*0.71*	**3.29**	*0.43*	**2.27**	*0.48*
fibronectin 1	FN1	ai262682	**2.91**	*1.05*	**1.94**	*0.79*	**2.92**	*0.41*	**1.97**	*0.58*
fibronectin 1	FN1	ai926509	**2.05**	*1.17*	**1.70**	*0.82*	**2.76**	*0.56*	**1.86**	*0.59*
fibronectin 1	FN1	ai832772	**2.13**	*0.53*	**1.67**	*0.59*	**2.62**	*0.35*	**1.54**	*0.51*
glypican 4	GPC4	ai368019	**2.53**	*0.83*	**1.94**	*1.17*	**2.99**	*0.96*	**1.09**	*1.03*
chondroitin sulfate proteoglycan 2	CSPG2	h52439	**2.40**	*1.55*	**2.38**	*1.01*	**4.38**	*0.80*	**3.63**	*0.91*
Latent TGF β binding protein 1	LTBP1	n93841	**2.46**	*0.73*	**2.00**	*0.80*	**2.04**	*0.48*	**2.16**	*0.71*
Cell signaling										
DNA-damage-inducible transcript 4	DDIT4	aa016188	**2.10**	*0.79*	**2.11**	*0.81*	**2.44**	*0.63*	**3.03**	*0.84*
transmembrane 4 superfamily member 1	TM4SF1	h38178	**3.35**	*0.66*	**2.42**	*0.43*	**2.13**	*0.73*	**1.71**	*1.35*
transmembrane 4 superfamily member 1	TM4SF1	ai189378	**3.29**	*0.95*	**2.19**	*0.54*	**2.24**	*0.73*	**1.67**	*1.43*
transmembrane 4 superfamily member 1	TM4SF1	ai911914	**2.51**	*1.13*	**2.07**	*0.55*	**1.80**	*1.10*	**1.74**	*1.12*
angiotensin II receptor, type 1	AGTR1	r01615	**3.52**	*0.93*	**2.27**	*0.78*	**1.97**	*0.94*	**2.89**	*1.76*
Cell growth / development										
mitogen-inducible gene 6	MIG-6	n39305	**1.75**	*0.29*	**1.08**	*0.32*	**2.03**	*0.38*	**3.07**	*0.41*
steroid-sensitive gene 1	URB	aa446024	**1.52**	*0.57*	**1.89**	*1.09*	**2.31**	*0.30*	**1.28**	*1.37**
steroid-sensitive gene 1	URB	aa024662	**1.61**	*0.80*	**1.78**	*1.09*	**2.41**	*0.33*	**1.13**	*1.31**
Transcription regulatory protein										
inhibitor of DNA binding 1	ID1	h63080	**1.59**	*0.61*	**1.38**	*0.55*	**1.18**	*0.37*	**2.76**	*0.92*
nuclear factor I/B	NFIB	aa047535	**1.63**	*0.53*	**2.49**	*0.79*	**1.22**	*0.63*	**1.08**	*1.67**
delta sleep-inducing peptide	DSIPI	n50052	**1.70**	*0.91*	**1.84**	*1.02*	**2.18**	*0.53*	**3.21**	*1.91*
Homeobox protein HOX-A5	HOXA5	n89758	**1.31**	*0.87*	**1.06**	*0.95*	**1.44**	*0.67*	**0.74****	*1.80**
Homeobox protein HOX-B6	HOXB6	aa427938	**1.60**	*0.78*	**1.10**	*0.68*	**2.26**	*0.31*	**0.89****	*1.06**
Other										
coiled-coil domain containing 2	CCDC2	w95494	**2.18**	*0.45*	**1.48**	*0.57*	**2.60**	*0.31*	**1.77**	*0.41*
copineVIII	CPNE8	ai188010	**2.14**	*0.70*	**1.72**	*0.44*	**1.62**	*0.45*	**1.21**	*0.51*
discs, large homolog-associated protein 1	DLGAP1	aa284288	**2.48**	*0.81*	**1.83**	*0.77*	**2.56**	*0.73*	**1.64**	*0.62*
FLJ00133 protein	SNED1	r72086	**2.20**	*1.69*	**1.08**	*1.36**	**1.51**	*0.81*	**1.24**	*2.38**
SEC13-like 1	SEC13L1	w72422	**2.64**	*0.65*	**1.93**	*0.77*	**3.09**	*0.41*	**2.15**	*0.39*
lysine hydroxylase	PLOD2	h97211	**2.60**	*0.84*	**1.57**	*0.85*	**2.37**	*1.16*	**1.62**	*1.07*
heterogeneous nuclear RNP M	HNRPM	w95488	**2.02**	*0.26*	**1.35**	*0.58*	**2.52**	*0.23*	**1.70**	*0.29*
Hypothetical protein MGC20262	MGC20262	aa055361	**2.11**	*0.89*	**1.26**	*0.75*	**3.24**	*0.42*	**1.31**	*2.03**
phosphoserine phosphatase	PSPH	n75009	**1.42**	*1.13*	**1.00**	*1.02**	**1.29**	*0.89*	**1.16**	*0.74*
KIAA1712	KIAA1712	w92618	**2.74**	*0.52*	**1.95**	*0.62*	**3.19**	*0.29*	**2.12**	*0.38*

All genes with a more than twofold upregulation in each of the four types of MSC vs HS68 are summarized in this table. Mean differential expression in 8 corresponding hybridizations is presented as log_2ratio (mean log_2ratio > 1). **(*) = mean ratio < SD;** (**) = less than twofold upregulation in CB-MSC-M3. Error estimate is presented as standard deviation (SD). FDR = 0.

HS68 cells. Analysis revealed a significantly higher representation of genes involved in morphogenesis and development in MSC. In contrast, a high percentage of those genes upregulated in HS68 fibroblasts were involved in homeostasis, vesicle formation, and metabolism (Table 4).

Differences in the gene expression profiles of MSC derived from AT, CB, and BM

We have analyzed differential expression of AT-MSC-M1, CB-MSC-M3, BM-MSC-M1, and BM-MSC-M2 by pairwise comparison. Various genes (between 197 and 1472 ESTs) were significantly higher ($p < 0.001$) and differentially expressed and the results are summarized in Figure 4.

Comparison with hematopoietic progenitor cells

Under the presumption that the differential potential of adult stem cells could be governed by unique molecular mechanisms, we have compared our data with our previous study on two fractions enriched in hematopoietic stem cells, namely $CD34^+/CD38^-$ cells (vs $CD34^+/CD38^+$ cells)

Table 3. Genes downiregulated (>fourfold) in all types of MSC vs human fibroblasts (HS68)

Gene name	Shortcut	Acc.No.	BM-MSC-M1		AT-MSC-M1		BM-MSC-M2		CB-MSC-M3	
			Log_2ratio±*SD*		Log_2ratio±*SD*		Log_2ratio±*SD*		Log_2ratio±*SD*	
Signal transduction / Cell signaling										
ankyrin repeat and SOCS box-containing	ASB5	ai349499	**−2.85**	*0.67*	**−3.64**	*1.05*	**−2.49**	*1.39*	**−3.28**	0.99
dickkopf homolog 1 (*Xenopus laevis*)	DKK1	n94525	**−4.18**	*1.08*	**−3.04**	*0.62*	**−3.66**	*1.13*	**−2.30**	*1.62*
inositol polyphosphate-4-phosphatase	INPP4B	r86720	**−2.08**	*0.91*	**−2.35**	*0.70*	**−2.33**	*0.46*	**−2.58**	*1.16*
protein tyrosine phosphatase, receptor B	PTPRB	h18633	**−3.11**	*1.72*	**−3.12**	*0.67*	**−2.54**	*0.89*	**−2.26**	*1.26*
interleukin 1, β	IL1B	w47101	**−3.17**	*1.07*	**−3.79**	*0.42*	**−3.15**	*0.82*	**−4.06**	*0.51*
secreted frizzled-related protein 1	SFRP1	aa150696	**−3.45**	*0.86*	**−3.86**	*1.24*	**−4.56**	*1.14*	**−4.73**	*1.01*
stathmin-like 2	STMN2	r19072	**−2.58**	*1.27*	**−2.70**	*3.00**	**−4.18**	*1.14*	**−5.19**	*0.86*
v-kit feline sarcoma viral oncogene	KIT	n26098	**−3.18**	*1.63*	**−3.63**	*0.95*	**−2.78**	*0.75*	**−2.51**	*1.06*
chemokine (C-C motif) receptor-like 1	CCRL1	aa400266	**−3.18**	*1.04*	**−3.05**	*0.50*	**−3.27**	*0.72*	**−4.04**	*0.45*
Protein metabolism										
matrix metalloproteinase 1 (interstitial)	MMP1	w49496	**−5.09**	*1.59*	**−2.51**	*1.52*	**−5.36**	*1.38*	**−5.34**	*1.30*
matrix metalloproteinase 10	MMP10	ai085155	**−2.29**	*0.62*	**−2.20**	*0.77*	**−2.69**	*0.61*	**−2.47**	*0.53*
matrix metalloproteinase 3	MMP3	w47091	**−6.15**	*0.97*	**−3.95**	*1.13*	**−6.98**	*0.77*	**−6.46**	*0.94*
Plasminogen activator inhibitor-2	SERPINB2	aa780136	**−3.71**	*1.23*	**−2.59**	*1.11*	**−4.32**	*1.10*	**−3.66**	*1.79*
Plasminogen activator inhibitor-2	SERPINB2	h82067	**−3.69**	*0.89*	**−2.55**	*0.88*	**−3.66**	*0.73*	**−3.31**	*1.04*
Ubiquitin-specific protease 53	USP53	af085848	**−2.85**	*0.97*	**−2.48**	*0.65*	**−2.35**	*0.79*	**−2.47**	*1.45*
Ubiquitin-specific protease 53	USP53	ai567034	**−2.84**	*0.31*	**−2.14**	*0.66*	**−2.89**	*0.77*	**−2.63**	*1.20*
lysyl oxidase-like 4	LOXL4	ai028603	**−3.71**	*0.66*	**−4.41**	*0.47*	**−2.32**	*0.46*	**−3.80**	*0.88*
Immune response										
Pregnancy-specific β-1-glycoprotein 1	PSG1	r68101	**−4.28**	*1.12*	**−3.59**	*0.69*	**−2.96**	*0.82*	**−2.54**	*1.37*
Pregnancy-specific β-1-glycoprotein 2	PSG2	h01004	**−3.77**	*1.57*	**−3.83**	*0.64*	**−2.96**	*0.47*	**−2.75**	*1.57*
Pregnancy-specific β-1-glycoprotein 3	PSG3	h12630	**−3.29**	*0.84*	**−3.46**	*0.64*	**−2.93**	*1.04*	**−2.28**	*1.59*
Pregnancy-specific β-1-glycoprotein 4	PSG4	r28356	**−2.48**	*0.70*	**−2.20**	*0.84*	**−2.50**	*0.91*	**−2.48**	*1.04*
Pregnancy-specific β-1-glycoprotein 4	PSG4	r26442	**−4.96**	*1.15*	**−4.68**	*0.77*	**−3.22**	*0.61*	**−3.08**	*1.58*
Pregnancy-specific β-1-glycoprotein 9	PSG9	t83938	**−2.93**	*1.44*	**−3.17**	*0.47*	**−2.22**	*0.50*	**−2.14**	*1.22*
Transcription factor										
T-box 5	TBX5	ai143430	**−2.63**	*1.19*	**−2.11**	*0.75*	**−2.59**	*0.94*	**−2.24**	*1.04*
transcription factor AP-2α	TFAP2A	r33626	**−4.10**	*1.36*	**−3.46**	*2.17*	**−3.16**	*1.06*	**−2.09**	*1.28*
Metabolism										
Aldehyde dehydrogenase 1A1	ALDH1A1	n75392	**−5.36**	*1.28*	**−5.42**	*0.57*	**−5.38**	*1.11*	**−3.89**	*3.66*
monooxygenase, DBH-like 1	MOXD1	aa424574	**−2.22**	*0.65*	**−3.13**	*0.83*	**−3.07**	*0.60*	**−3.13**	*0.98*
Other										
Adenomatosis polyposis coli down-reg	APCDD1	r10703	**−2.67**	*1.29*	**−2.59**	*0.70*	**−2.42**	*0.48*	**−2.87**	*0.93*
chromosome 10 open reading frame 48	C10orf48	aa142923	**−3.89**	*2.19*	**−4.52**	*1.24*	**−2.37**	*1.39*	**−4.03**	*1.37*
chromosome 8 open reading frame 4	C8orf4	h16793	**−3.74**	*0.59*	**−4.00**	*0.42*	**−3.86**	*1.33*	**−3.25**	*2.25*
hypothetical protein FLJ11259	FLJ11259	w67782	**−3.03**	*0.49*	**−4.08**	*0.29*	**−2.46**	*0.62*	**−2.34**	*1.19*
normal mucosa of esophagus specific 1	NMES1	aa620995	**−2.10**	*1.94*	**−2.36**	*2.16*	**−2.43**	*0.30*	**−2.15**	*2.55**
RGM domain family, member B	RGMB	n29591	**−2.01**	*2.47**	**−2.77**	*1.25*	**−2.23**	*0.87*	**−2.53**	*0.51*

All genes with a more than fourfold downregulation in each of the four types of MSC vs HS68 are summarized in this table. Mean differential expression of 8 corresponding hybridizations is presented as log_2ratio (mean log_2ratio< −2). Error estimate is presented as standard deviation (SD). **(*) = mean ratio < SD**. FDR = 0.

and on the slow-dividing fraction in $CD34^+/CD38^-$ cells (SDF; as compared to the fast-dividing fraction, FDF) [39]. ID1, NFIB, and ECM2 were higher expressed in all preparations of MSC and in $CD34^+/CD38^-$ cells. FN1, CSPG2, and DLGAP1 were higher expressed in all MSC and in the SDF. In contrast, none of the ESTs was in the overlap of all MSC and the more committed progenitor fractions of $CD34^+/CD38^+$ cells or FDF.

Confirmation of differential expression by RT-PCR

Differential gene expression of 11 regulated genes and 3 housekeeping genes was determined by real-time PCR as presented in Figure 5. Specific amplification was proven by melting curve analysis and conventional agarose gel electrophoresis, whereas products for TBX5 and NCAM1 were only amplified in HS68 samples but not in BM-MSC-M1. The tendency of differential expression in BM-MSC-M1 vs HS68 was consistent between microarray data and semi-quantitative RT-PCR analysis in all genes tested. Furthermore, RT-PCR confirmed differential expression as observed between the four different MSC sources.

Discussion

Preparative protocols for the acquisition, separation, in vitro cultivation, and expansion of MSC have been extremely

Table 4. Classification of differentially regulated genes according to gene ontology terms

Go ID	Term	Upregulated in MSC (total 160 genes)	Upregulated in HS68 (total 418 genes)	*p*
Categories over-represented in MSC				
0000122	negative regulation of Pol II promoter	2.5%	0.0%	0.01
0005634	nucleus	30.6%	22.2%	0.01
0002009	morphogenesis of an epithelium	3.1%	0.5%	0.02
0007160	cell-matrix adhesion	2.5%	0.2%	0.02
0007606	sensory perception of chemical stimulus	2.5%	0.2%	0.02
0048468	cell development	2.5%	0.2%	0.02
0009948	anterior/posterior axis specification	1.9%	0.0%	0.02
0045765	regulation of angiogenesis	1.9%	0.0%	0.02
0000902	cellular morphogenesis	6.3%	2.6%	0.03
0000165	MAPKKK cascade	3.8%	1.2%	0.04
0031012	extracellular matrix	8.8%	5.7%	0.06
0007275	development	33.1%	31.6%	0.07
Categories over-represented in HS68 fibroblasts				
0016192	vesicle-mediated transport	0.6%	6.7%	0.00
0005764	lysosome	0.0%	3.6%	0.01
0042592	homeostasis	0.0%	3.3%	0.01
0044267	cellular protein metabolism	19.4%	27.0%	0.01
0007267	cell-cell signaling	3.8%	8.6%	0.02
0008565	protein transporter activity	0.0%	2.9%	0.02
0016023	cytoplasmic vesicle	0.6%	3.8%	0.02
0006897	endocytosis	0.0%	2.6%	0.03
0006796	phosphate metabolism	5.0%	9.6%	0.03
0006950	response to stress	9.4%	14.6%	0.03
0044260	cellular macromolecule metabolism	22.5%	28.5%	0.03
0000074	regulation of cell cycle	3.1%	6.7%	0.04
0007399	neurogenesis	5.0%	8.6%	0.05

Differentially regulated genes (comparison of all MSC vs HS68 fibroblasts) were classified by GeneOntology categories. Filter criteria for selected genes: mean $\log_2$ratio $>$ SD (in data of 32 co-hybridizations). Functional classification according to GO-terms was assigned to 160 genes that were upregulated in MSC and 418 genes upregulated in HS68. Probability (p) for representation in functional groups was determined by hypergeometric distribution.

heterogeneous. Phenotypically, the starting cell material has been defined by a panel of surface markers, by subsequent adherence to plastic surface, and by their propensity to give rise to adipocytes, chondrocytes, and osteoblasts [7,12,23–25,44,45]. Cell preparations fulfilling these characteristics have been derived from bone marrow, adipose tissue, and umbilical cord blood. According to surface antigen markers, human fibroblast cell lines (HS68 and NHDF) displayed an identical phenotype. Thus, a phenotypic analysis using surface markers is not sufficient to define MSC.

Genomic studies have provided another dimension for a detailed understanding of multipotent stem cells. Several authors have analyzed gene expression profiles of MSC and these studies have focused on 1) changes induced in the course of in vitro differentiation to osteogenic [46], chondrogenic [47,48], or adipogenic lineages [49,50], 2) direct comparison of gene expression profiles in different populations of MSC [15,51,52], and 3) estimation of the absolute expression levels in MSC [53–55]. A systematic comparison of MSC with cells that represent terminally differentiated cells of mesodermal derivation has not yet been described.

In this study we have analyzed the global gene expression profiles of MSC preparations with our Human Genome Microarray (51,144 different ESTs) [39]. Differential gene expression was determined in relation to HS68 fibroblasts. Initial analysis has demonstrated a consistent upregulation of 25 well-characterized genes in all MSC preparations derived from different tissues or cultivated under different culture conditions. We have therefore focused on these genes and their role in MSC. Among these, fibronectin 1 (FN1) was eminently upregulated. Other authors have demonstrated that FN1 was among the highest-expressed genes in BM-MSC by microsage analysis and the highest-expressed gene in EST sequencing analysis [53–55]. We have previously demonstrated that FN1 was higher expressed in the slow-dividing fraction of $CD34^+/CD38^-$ cells that is enriched in hematopoietic stem cells [39]. This glycoprotein plays a fundamental role in the organization and composition of the extracellular matrix and cell-matrix adhesion sites [56], and since MSC can be isolated by adherent growth on culture wells coated with fibronectin [21] this cell-matrix interaction seems to be of particular importance for the growth of MSC. Other extracellular

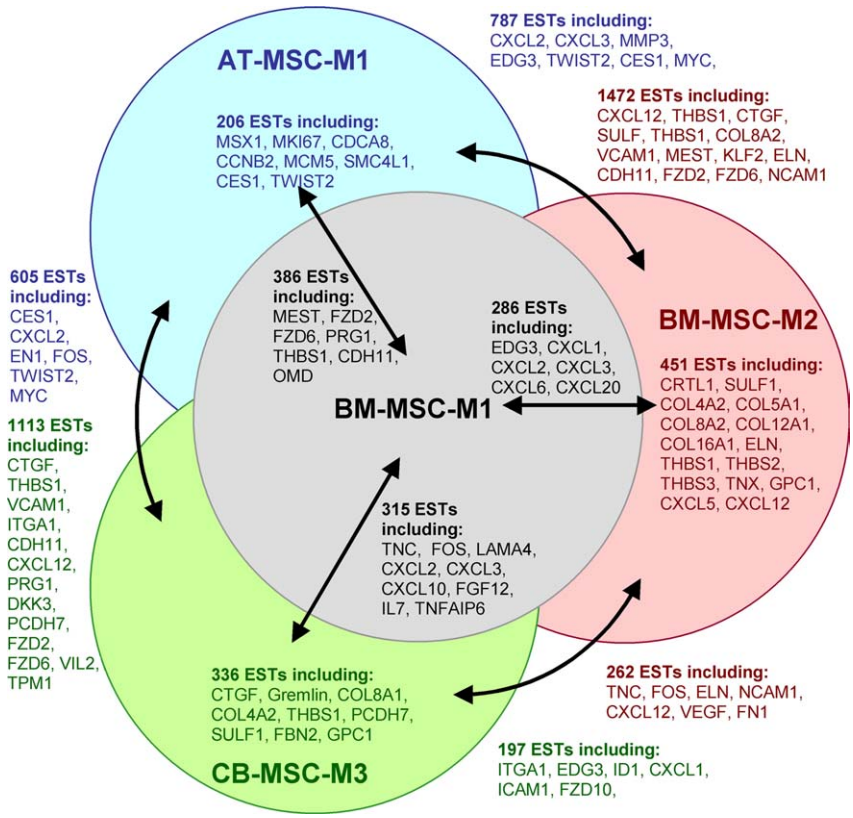

Figure 4. Pairwise comparison of MSC derived from AT, CB, and BM. Gene expression of AT-MSC-M1, CB-MSC-M1, BM-MSC-M2, and BM-MSC-M1 was compared pairwise. The numbers of ESTs that revealed highly significant upregulation in the corresponding cell types are presented ($p < 0.001$). Gene symbols of selected genes are provided.

matrix proteins that were higher expressed in all fractions of MSC included GPC4, LTBP1, ECM2, and CSPG2 and these results indicate that the composition of extracellular matrix proteins plays an essential role and organization of extracellular matrix might be characteristic for MSC. Transcription factors that were higher expressed in MSC included nuclear factor I/B (NFIB) and homeobox genes HOXA5 and HOXB6, and they play an important role in the regulation of mammalian development [57,58]. Inhibitor of differentiation/DNA binding ID1 and ID4 can form heterodimers with members of the basic HLH family of transcription factors, thereby regulating cell growth, senescence, and differentiation [59]. ID1, ECM2, and NFIB have been upregulated in our previous study on hematopoietic $CD34^+/CD38^-$ cells as compared to more committed $CD34^{+/}CD38^+$ cells [39]. Under the presumption that self-renewal and differentiation are governed by a shared molecular mechanism, other authors have found similar gene expression patterns in various types of stem cells [60–63]. Comparison with the corresponding datasets revealed that genes commonly upregulated in both MSC and hematopoietic progenitor cells in our studies were not coherently upregulated in other types of embryonic or adult stem cells. Thus it is unlikely that these markers are common ''stemness'' markers, but they might be valuable for identifying MSC.

Obviously, the 25 genes that were differentially upregulated in the MSC preparations compared to fibroblasts could represent good targets for identifying novel MSC markers. For practical reasons, candidate genes that are highly expressed or that are localized on the cell surface in analogy to CD34 or CD133 for hematopoietic stem cells would be desirable. In contrast, transcription factors and regulators of signal transduction are often scarcely expressed and the use of extracellular proteins is unfavorable for quality control purposes. Our results indicate that a single marker might not be adequate, but rather a combination of surface antigens and gene expression parameters might be necessary to specifically identify multipotent MSC. Nevertheless, our data have provided the basis for a selection of upregulated genes that might serve as a quality control of MSC at a genomic level. As demonstrated in our study, a comprehensive and comparative analysis with other types of stem cell preparations, as well as a variety of terminally differentiated cell types, are necessary to define a subset of reliable molecular markers.

Many genes of the Wnt pathway were differentially expressed in MSC populations as compared to fibroblasts.

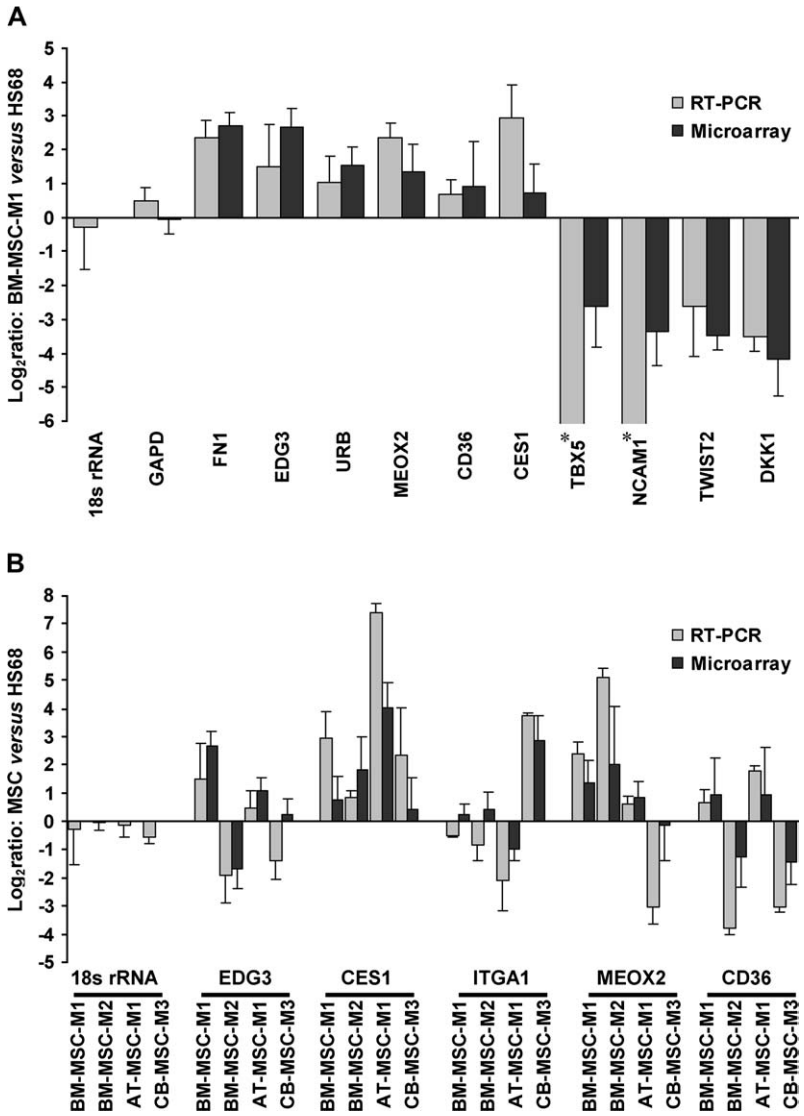

Figure 5. Confirmation of differential expression by RT-PCR. Semiquantitative light cycler RT-PCR was used to analyze differential gene expression of selected genes in relation to ubiquitin. 18s rRNA and GAPDH were used as additional housekeeping genes. The two methods demonstrated good correlation of differential gene expression of BM-MSC-M1 vs HS68 **(A)** as well as differential expression between different types of MSC **(B)**. (*) = specific RT-PCR product of TBX5 and NCAM1 was amplified in HS68 but not in BM-MSC-M1.

Analysis of gene expression profiles does not reflect the transient regulations in signal cascades but the repertoire of upregulated genes might help to highlight important pathways. The Wnt pathway has previously been shown to play a role in differentiation of neural systems [64], skeletal muscle [65], cardiac cells [66], endoderm [67], cartilage [68], and limbs [69]. Here we demonstrate that the inhibitors of this pathway dickkopf-1 and -3 (DKK-1, -3) and secreted frizzeled related proteins-1 and -4 (SFRP-1, -4) are downregulated in our MSC preparations. In contrast, FZD1 as well as several effectors of the canonical and non-canonical pathway were higher expressed in all preparations of MSC. Gregory et al. have recently demonstrated that DKK1 expression was decreased in human adult stem cells from the bone marrow as they entered the stationary phase [70]. Furthermore they have provided evidence that DKK1 is required for reentry into the cell cycle and demonstrated that the lower expression of DKK1 in the stationary phase induced an enhanced expression of VCAM1. In our study all cells were harvested upon reaching 80% confluency and thus DKK1 might be downregulated while VCAM1 might be upregulated, in contrast to growth in the exponential phase. In accordance with observations from other authors, our results have confirmed that the Wnt pathway plays a significant role also in MSC [71].

Comparison of different MSC preparations by hierarchical cluster analysis demonstrated a very close resemblance of the profiles among all the four donor samples for each

tissue or for each culture condition. This indicated that using standardized procedures, we were able to reproducibly establish a relative homogeneous MSC population. We have therefore provided evidence that genotypic analysis could represent a new dimension for identifying and defining MSC. Flow cytometry and immunofluorescent microscopy detecting several proteins that have been shown to be differentially expressed (e.g., fibronectin, cadherin 11, N-cadherin, VE-cadherin) have verified our present observation and might represent common denominators for specific homogeneous subsets of MSC.

In contrast, BM-MSC isolated from the same donor under different culture conditions did not cluster together. Lee et al. have previously described that culture conditions have a significant impact on the gene expression profile [52]. At least some of these differences in gene expression could be due to the direct influence exercised by specific components in the culture media, in this case 10% FCS. Morphological differences and the large variety of differentially expressed genes suggest that culture conditions affect the selection of specific cell populations with different potentials. Isolation of MSC is primarily based on plastic adherence and growth under specific culture conditions and hence, it is not surprising that culture media and growth factors might play a significant role in the selection of cell populations.

It has been demonstrated that MSC populations can be isolated from various human tissues [6–12]. Whether these cells are closely related to each other on a molecular basis remains yet unresolved. Jiang et al. suggested that MSC from bone marrow, muscle, and brain have almost identical gene expression profiles [15]. Furthermore, Lee et al. have reported that gene expression profiles of BM-MSC and AT-MSC were similar [52]. In this study, we have demonstrated significant differences in the global gene expression patterns of MSC from AT, CB vs BM. Several genes involved in mesodermal differentiation were differentially expressed. For example, mesoderm-specific transcript homolog (MEST) that is predominantly expressed in the mesodermal lineage of the mouse embryo was highly expressed in BM-MSC-M1 and BM-MSC-M2, whereas the highest expressions of BMP antagonist 1 (CKTSF1B1 or gremlin 1) and connective tissue growth factor (CTGF) were found in CB-MSC-M3. Gremlin 1 is expressed in embryonic mesenchymal cells and seems to play an important role in epithelial-mesenchymal feedback signaling of organogenesis [72] while connective tissue growth factor (CTGF) displays multiple functions in mesenchymal cells, including the promotion of proliferation and normal osteoblast and chondrocyte differentiation [73,74]. These results implied that potentials of mesodermal development might be different in these MSC preparations. Ki-67, cell division cycle associated 8 (CDCA8), and cyclin B2 (CCNB2) were higher expressed in AT-MSC-M1 than in BM-MSC-M1, and this indicated that MSC derived from AT could have a higher proliferative activity. Indeed, Lee et al. have demonstrated that AT-MSC multiplied faster for up to 20 passages in culture as compared to BM-MSC [52].

The gene expression profiles of MSC could reflect on their tissues of origin. Functional classification of differentially expressed genes according to the GeneOntology convention has demonstrated higher expression of genes in the categories ossification, skeletal development, and bone remodeling in BM-MSC-M2; extracellular matrix in BM-MSC-M1, BM-MSC-M2, and CB-MSC-M3; and triacylglycerol biosynthesis in BM-MSC-M1 and AT-MSC-M1 (determined by a statistical test under the hypergeometric distribution; results not demonstrated), but overall the observed pattern did not reflect molecular characteristics of the tissue that was initially used for the isolation. To determine the role of individual genes on cell fate and differentiation potential, additional functional studies will be necessary for selected genes and are concurrently under way.

MSC and some of the equivalent cell lines seem to hold promise for future stem cell–based therapy strategies and for tissue engineering. A major obstacle is the lack of definition and standardization of MSC. Our results indicated that homogeneous cell populations could be established under standardized operating procedures and the resulting cell lines are reproducible, at least according to genotypic parameters, whereas there are significant differences in the transcriptome of MSC isolated from AT, CB, or BM. Whether these differences have significant impact on their functions as adult stem cells for regenerative medicine remains to be defined. Nevertheless, 25 overlapping, upregulated genes observed in all MSC preparations is a remarkable finding and this might provide the foundation for establishing guidelines for the molecular identification and definition of MSC. This will in turn contribute to establish a reliable quality control system for clinical applications.

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References

1. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 1997;276:71–74.
2. Prockop DJ, Gregory CA, Spees JL. One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. Proc Natl Acad Sci U S A. 2003;100(suppl 1):11917–11923.

3. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002;418: 41–49.
4. Petersen BE, Bowen WC, Patrene KD, et al. Bone marrow as a potential source of hepatic oval cells. Science. 1999;284:1168–1170.
5. Schwartz RE, Reyes M, Koodie L, et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest. 2002;109:1291–1302.
6. Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol. 1966;16: 381–390.
7. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143–147.
8. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001;7: 211–228.
9. Bieback K, Kern S, Kluter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells. 2004;22:625–634.
10. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol. 2000;109:235–242.
11. Goodwin HS, Bicknese AR, Chien SN, et al. Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. Biol Blood Marrow Transplant. 2001; 7:581–588.
12. Kogler G, Sensken S, Airey JA, et al. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med. 2004;200:123–135.
13. Zvaifler NJ, Marinova-Mutafchieva L, Adams G, et al. Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res. 2000; 2:477–488.
14. Kuznetsov SA, Mankani MH, Gronthos S, et al. Circulating skeletal stem cells. J Cell Biol. 2001;153:1133–1140.
15. Jiang Y, Vaessen B, Lenvik T, et al. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol. 2002;30:896–904.
16. Javazon EH, Beggs KJ, Flake AW. Mesenchymal stem cells: paradoxes of passaging. Exp Hematol. 2004;32:414–425.
17. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow–derived mesenchymal progenitor cells. Exp Cell Res. 1998;238:265–272.
18. Mackay AM, Beck SC, Murphy JM, et al. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng. 1998;4:415–428.
19. Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci. 2000;113(Pt 7):1161–1166.
20. Ryden M, Dicker A, Gotherstrom C, et al. Functional characterization of human mesenchymal stem cell–derived adipocytes. Biochem Biophys Res Commun. 2003;311:391–397.
21. Reyes M, Lund T, Lenvik T, et al. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood. 2001; 98:2615–2625.
22. Hauner H, Entenmann G, Wabitsch M, et al. Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. J Clin Invest. 1989;84: 1663–1670.
23. Tocci A, Forte L. Mesenchymal stem cell: use and perspectives. Hematol J. 2003;4:92–96.
24. Haynesworth SE, Baber MA, Caplan AI. Cell surface antigens on human marrow–derived mesenchymal cells are detected by monoclonal antibodies. Bone. 1992;13:69–80.
25. Gronthos S, Franklin DM, Leddy HA, et al. Surface protein characterization of human adipose tissue–derived stromal cells. J Cell Physiol. 2001;189:54–63.
26. Hou L, Cao H, Wang D, et al. Induction of umbilical cord blood mesenchymal stem cells into neuron-like cells in vitro. Int J Hematol. 2003;78:256–261.
27. Quarto R, Mastrogiacomo M, Cancedda R, et al. Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med. 2001;344:385–386.
28. Fuchs JR, Hannouche D, Terada S, Vacanti JP, Fauza DO. Fetal tracheal augmentation with cartilage engineered from bone marrow–derived mesenchymal progenitor cells. J Pediatr Surg. 2003;38:984–987.
29. Gangji V, Hauzeur JP, Matos C, et al. Treatment of osteonecrosis of the femoral head with implantation of autologous bone-marrow cells. A pilot study. J Bone Joint Surg Am. 2004;86-A:1153–1160.
30. Horwitz EM, Prockop DJ, Fitzpatrick LA, et al. Transplantability and therapeutic effects of bone marrow–derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med. 1999;5: 309–313.
31. Horwitz EM, Gordon PL, Koo WK, et al. Isolated allogeneic bone marrow–derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proc Natl Acad Sci U S A. 2002;99:8932–8937.
32. Koc ON, Day J, Nieder M, et al. Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH). Bone Marrow Transplant. 2002;30: 215–222.
33. Chen SL, Fang WW, Ye F, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol. 2004;94:92–95.
34. Deb A, Wang S, Skelding KA, et al. Bone marrow–derived cardiomyocytes are present in adult human heart: A study of gender-mismatched bone marrow transplantation patients. Circulation. 2003; 107:1247–1249.
35. Kadereit S, Deeds LS, Haynesworth SE, et al. Expansion of LTC-ICs and maintenance of p21 and BCL-2 expression in cord blood $CD34^+/CD38^-$ early progenitors cultured over human MSCs as a feeder layer. Stem Cells. 2002;20:573–582.
36. Zhang Y, Li C, Jiang X, et al. Human placenta–derived mesenchymal progenitor cells support culture expansion of long-term culture-initiating cells from cord blood $CD34^+$ cells. Exp Hematol. 2004;32: 657–664.
37. Fouillard L, Bensidhoum M, Bories D, et al. Engraftment of allogeneic mesenchymal stem cells in the bone marrow of a patient with severe idiopathic aplastic anemia improves stroma. Leukemia. 2003;17: 474–476.
38. Ho AD, Punzel M. Hematopoietic stem cells: can old cells learn new tricks? J Leukoc Biol. 2003;73:547–555.
39. Wagner W, Ansorge A, Wirkner U, et al. Molecular evidence for stem cell function of the slow-dividing fraction among human hematopoietic progenitor cells by genome-wide analysis. Blood. 2004;104: 675–686.
40. Richter A, Schwager C, Hentze S, et al. Comparison of fluorescent tag DNA labeling methods used for expression analysis by DNA microarrays. Biotechniques. 2002;33:620–628, 630.
41. Brazma A, Hingamp P, Quackenbush J, et al. Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. Nat Genet. 2001;29:365–371.
42. Rossmann H, Bachmann O, Vieillard-Baron D, Gregor M, Seidler U. Na+/. Gastroenterology. 1999;116:1389–1398.
43. Niemeyer P, Krause U, Fellenberg J, et al. Evaluation of mineralized collagen and α-tricalcium phosphate as scaffolds for tissue engineering of bone using human mesenchymal stem cells. Cells Tissues Organs. 2004;177:68–78.
44. Aust L, Devlin B, Foster SJ, et al. Yield of human adipose-derived adult stem cells from liposuction aspirates. Cytotherapy. 2004;6: 7–14.

45. Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002;13:4279–4295.
46. Doi M, Nagano A, Nakamura Y. Genome-wide screening by cDNA microarray of genes associated with matrix mineralization by human mesenchymal stem cells in vitro. Biochem Biophys Res Commun. 2002;290:381–390.
47. Sekiya I, Vuoristo JT, Larson BL, Prockop DJ. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc Natl Acad Sci U S A. 2002;99:4397–4402.
48. Qi H, Aguiar DJ, Williams SM, et al. Identification of genes responsible for osteoblast differentiation from human mesodermal progenitor cells. Proc Natl Acad Sci U S A. 2003;100:3305–3310.
49. Sekiya I, Larson BL, Vuoristo JT, Cui JG, Prockop DJ. Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs). J Bone Miner Res. 2004;19:256–264.
50. Nakamura T, Shiojima S, Hirai Y, et al. Temporal gene expression changes during adipogenesis in human mesenchymal stem cells. Biochem Biophys Res Commun. 2003;303:306–312.
51. Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol. 1998; 176:57–66.
52. Lee RH, Kim B, Choi I, et al. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. Cell Physiol Biochem. 2004;14:311–324.
53. Tremain N, Korkko J, Ibberson D, et al. MicroSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages. Stem Cells. 2001;19:408–418.
54. Jia L, Young MF, Powell J, et al. Gene expression profile of human bone marrow stromal cells: high-throughput expressed sequence tag sequencing analysis. Genomics. 2002;79:7–17.
55. Wieczorek G, Steinhoff C, Schulz R, et al. Gene expression profile of mouse bone marrow stromal cells determined by cDNA microarray analysis. Cell Tissue Res. 2003;311:227–237.
56. Sottile J, Hocking DC. Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions. Mol Biol Cell. 2002;13:3546–3559.
57. Chaudhry AZ, Lyons GE, Gronostajski RM. Expression patterns of the four nuclear factor I genes during mouse embryogenesis indicate a potential role in development. Dev Dyn. 1997;208: 313–325.
58. Giampaolo A, Felli N, Diverio D, et al. Expression pattern of HOXB6 homeobox gene in myelomonocytic differentiation and acute myeloid leukemia. Leukemia. 2002;16:1293–1301.
59. Sikder HA, Devlin MK, Dunlap S, Ryu B, Alani RM. Id proteins in cell growth and tumorigenesis. Cancer Cell. 2003;3:525–530.
60. Ivanova NB, Dimos JT, Schaniel C, et al. A stem cell molecular signature. Science. 2002;298:601–604.
61. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. ''Stemness'': transcriptional profiling of embryonic and adult stem cells. Science. 2002;298:597–600.
62. Li L, Akashi K. Unraveling the molecular components and genetic blueprints of stem cells. Biotechniques. 2003;35:1233–1239.
63. Pyle AD, Donovan PJ, Lock LF. Chipping away at ''stemness.''. Genome Biol. 2004;5:235.
64. Packard M, Koo ES, Gorczyca M, et al. The Drosophila Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. Cell. 2002;111:319–330.
65. Petropoulos H, Skerjanc IS. β-catenin is essential and sufficient for skeletal myogenesis in P19 cells. J Biol Chem. 2002;277:15393–15399.
66. Pandur P, Lasche M, Eisenberg LM, Kuhl M. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. Nature. 2002;418:636–641.
67. Liu T, Lee YN, Malbon CC, Wang HY. Activation of the β-catenin/Lef-Tcf pathway is obligate for formation of primitive endoderm by mouse F9 totipotent teratocarcinoma cells in response to retinoic acid. J Biol Chem. 2002;277:30887–30891.
68. Fischer L, Boland G, Tuan RS. Wnt signaling during BMP-2 stimulation of mesenchymal chondrogenesis. J Cell Biochem. 2002;84:816–831.
69. Hartmann C, Tabin CJ. Wnt-14 plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. Cell. 2001;104:341–351.
70. Gregory CA, Singh H, Perry AS, Prockop DJ. The Wnt signaling inhibitor dickkopf-1 is required for reentry into the cell cycle of human adult stem cells from bone marrow. J Biol Chem. 2003;278:28067–28078.
71. Etheridge SL, Spencer GJ, Heath DJ, Genever PG. Expression profiling and functional analysis of wnt signaling mechanisms in mesenchymal stem cells. Stem Cells. 2004;22:849–860.
72. Michos O, Panman L, Vintersten K, et al. Gremlin-mediated BMP antagonism induces the epithelial-mesenchymal feedback signaling controlling metanephric kidney and limb organogenesis. Development. 2004;131:3401–3410.
73. Luo Q, Kang Q, Si W, et al. Connective tissue growth factor (CTGF) is regulated by Wnt and BMP signaling in osteoblast differentiation of mesenchymal stem cells. J Biol Chem. 2004;279:55958–55968.
74. Yosimichi G, Kubota S, Hattori T, et al. CTGF/Hcs24 interacts with the cytoskeletal protein actin in chondrocytes. Biochem Biophys Res Commun. 2002;299:755–761.