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Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146^{+} perivascular cells and fibroblasts

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Objective. **The relationship of multipotent mesenchymal stromal cells (MSC) with pericytes and fibroblasts has not been established thus far, although they share many markers of primitive marrow stromal cells and the osteogenic, adipogenic, and chondrogenic differentiation potentials.**

Materials and Methods. **We compared MSCs from adult or fetal tissues, MSC differentiated in vitro, fibroblasts and cultures of retinal pericytes obtained either by separation with anti-CD146 or adhesion. The characterizations included morphological, immunophenotypic, gene-expression profile, and differentiation potential.**

Results. **Osteogenic, adipocytic, and chondrocytic differentiation was demonstrated for MSC, retinal perivascular cells, and fibroblasts. Cell morphology and the phenotypes defined by 22 markers were very similar. Analysis of the global gene expression obtained by serial analysis of gene expression for 17 libraries and by reverse transcription polymerase chain reaction of 39 selected genes from 31 different cell cultures, revealed similarities among MSC, retinal perivascular cells, and hepatic stellate cells. Despite this overall similarity, there was a heterogeneous expression of genes related to angiogenesis, in MSC derived from veins, artery, perivascular cells, and fibroblasts. Evaluation of typical pericyte and MSC transcripts, such as NG2, CD146, CD271, and CD140B on CD146 selected perivascular cells and MSC by real-time polymerase chain reaction confirm the relationship between these two cell types. Furthermore, the inverse correlation between fibroblast-specific protein-1 and CD146 transcripts observed on pericytes, MSC, and fibroblasts highlight their potential use as markers of this differentiation pathway.**

Conclusion. **Our results indicate that human MSC and pericytes are similar cells located in the wall of the vasculature, where they function as cell sources for repair and tissue maintenance, whereas fibroblasts are more differentiated cells with more restricted differentiation potential.**

The denomination "mesenchymal stem cells" has been used to define spindle-like plastic adherent cells that can be expanded for more than 50 cell doublings without signs of senescence, and with osteogenic, chondrogenic, and adipogenic potential [1]. In an attempt to avoid applying the term *stem cell* to such heterogeneous populations, they have been named multipotent mesenchymal stromal cells [2] because the definition of a stem cell is neither firmly established nor universally accepted [3].

Multipotent mesenchymal stromal cells (MSC) are obtained from a variety of adult and fetal tissues, including cord blood [4], dental pulp [5], adipose tissue [6], skin [7], muscle [8], liver [9], synovium [10], pancreas [11], lung [12], amniotic fluid [13], placenta [14], peripheral blood [15], and others.

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Recent experimental evidence shows that the vessel wall contains multipotential stem cells that could be directly involved in vasculogenesis, atherosclerosis, and fibrosis [16,17]. In fact, the presence of stem-like cells in the wall of the vasculature was noticed for the first time 39 years ago [18]. For instance, pericytes, which were originally defined by their morphology and close contact to endothelial cells, can differentiate into other cell types, including osteoblasts, chondrocytes, adipocytes, fibroblasts, myofibroblasts, and smooth muscle cells (SMC) [19,20], a property shared by MSC [21,22]. Presently, no "pan-specific" marker is available to define pericyte phenotype unambiguously. Commonly used markers such as CD146, NG2, desmin, α-smooth-muscle actin and the regulator of G-protein signaling RGS5 label the heterogeneous pericyte population [23–25].

Fibroblasts, classically defined as mesenchymal-derived spindle-shaped cells that synthesize the major interstitial collagens [26] are also capable of originating myofibroblasts, contractile cells that express α-SMC actin and other mesenchymal markers [27]. It must be noted that both fibroblast-specific protein-1 (FSP-1) and S100A4 are two names for the same protein, which has been considered a specific fibroblast marker [28,29].

The differences between myofibroblasts and SMC are not well-defined [30]; furthermore, myofibroblasts are actively involved in the function of the vasculature as well as in pathologic processes, such as pulmonary, hepatic, and renal fibrosis [31–33]. Particularly in the liver, a population named hepatic stellate cells can differentiate into myofibroblasts, which plays an important role in liver fibrogenesis [34]. Additionally, hepatic stellate cells have been considered the hepatic pericyte and experimental evidence suggest that it could be derived from bone marrow (BM) MSC [35].

Thus, despite their similarities, a further understanding of how the so-called MSC relates to pericytes, fibroblasts, myofibroblasts, SMC and other differentially named cells such as hepatic stellate cells, has important implications in basic cell biology and in cell therapies.

We have isolated MSC from the subendothelial layer of the umbilical cord vein (UCV) [36] and compared their gene-expression profile with that of MSC from BM. The profiles were very similar, suggesting that these cells are basically the same, although there are small differences that probably reflect a specialized gene program related to their anatomic localization. For instance, MSC from the BM and UCV overexpress genes related to osteogenesis or to angiogenesis, respectively [37,38]. Further, we isolated MSC from saphena vein [39], and in this work we present the isolation and characterization of MSC from diverse human tissues.

Based on this initial evidence, we postulated that MSC with similar gene-expression profiles are widely distributed in the human body and that these cells are closely related to pericytes and fibroblasts. In order to test this hypothesis, we obtained the transcriptome of cultured retinal perivascular cells by serial analysis of gene expression (SAGE), to establish their relationship with our previously reported transcriptomes of MSC derived from BM and UCV, as well as with transcriptomes of fibroblasts, SMCs, hepatic stellate cells, and myofibroblasts derived from hepatic stellate cells. In addition, MSC isolated from various tissues were compared with other cell types, including pericytes and fibroblasts, based on their morphological features, differentiation potential, immunophenotypic profile, and expression of selected genes by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) or real-time PCR.

Materials and methods

Use of human cells in these experiments was approved by the institutional research review committee.

Fetal and adult tissues

Fetal tissues were obtained from aborted fetuses during autopsy. Fragments of 0.5 to 1.0 cm of the carotid, fascia of the abdominal rectus muscle, gonad, liver, and skin were placed in phosphate-buffered saline containing 0.2% glucose (Merck, Rio de Janeiro, Brazil) and 0.2% albumin (Baxter Healthcare Co, Glendale, CA, USA). Normal skin and adipose tissue samples of adults were obtained at diagnostic biopsies. BM aspirates were obtained from donors during collection for BM transplantation. Small segments of the saphena vein were obtained from patients submitted to saphenectomy for bypass heart surgery. Segments of human retina were obtained at autopsy. Umbilical cord blood was obtained from term deliveries and collected on sodium citrate, as described previously [40].

Culture of fetal and adult tissue MSC

Cell suspensions were obtained by mechanical disruption followed by enzymatic digestion of the fetal and adult tissues, as described previously [39]. Cells were cultured at a concentration of 2 to 4 × 10^5 cells/mL in 25 cm^2 flasks and maintained in α-minimum essential medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 15% fetal calf serum (HyClone, Logan, UT, USA), 2 mmol/L L-glutamine and 100 U penicillin/streptomycin (Sigma, St Louis, MO, USA). Nonadherent cells were removed after 72 hours and formation of small colonies was observed 3 to 4 days later. The plastic-adherence multipotent MSCs from third to fifth passages were used in the experiments.

Culture of umbilical cord blood and BM MSC

Mononuclear cells were separated by Ficoll-Hypaque from about 80 mL umbilical cord blood or 10 mL BM. MSCs were isolated by plastic adherence as described previously [38] and cultured as mentioned previously.

Pericytes

Pericytes were isolated from human retina from fetal and adult tissues as described previously [41]. At first passage, cells from fetal tissues were labeled with anti-CD146 and sorted using FACSAria flow cytometer (Becton-Dickinson, San Jose, CA, USA) in order to obtain a pure preparation of pericytes. For the isolation of retinal pericytes from adult, cells were grown on plastic with 20%

fetal bovine serum. Pericytes were used for experiments at the third passage.

Culture of fibroblasts

Primary cultures of human fibroblasts were obtained from adult abdominal skin, fetal muscle fascia, and foreskin tissues, as described previously [42]. Briefly, tissues were minced into 1- to 2-mm pieces and allowed to adhere to 75-cm^2 culture flasks. Cells were cultured in RPMI with 10% fetal calf serum, 2 mM L-glutamine and 100 UI/mL penicillin/streptomycin (37°C, 5% CO_2). After the 3rd passage for skin and muscle fascia, and at 11th and 17th passage for foreskin, separate aliquots were used for experiments. We used also the commercial human fetus skin fibroblast cell line CCD-27Sk from ATCC (cat. no. CRL-1475).

Human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated as originally described [43] and placed at a concentration of 10^5 cells/mL in TC199 medium with 20% fetal calf serum, 2 mM L-glutamine, 100 U penicillin/streptomycin, 0.05 μg/mL vascular endothelial growth factor (Sigma-Aldrich), 1 μg/mL hydrocortisone (Sigma), and 100 U/mL sodium heparin (Organon-Teknika, São Paulo, Brazil). Cells at the third passage were characterized phenotypically and RNA was extracted.

Differentiation capacity of multipotent MSCs, pericytes, and fibroblasts

Adipogenic, osteogenic, and chondrogenic differentiation evaluation were performed as described previously [36]. Basically, after incubation with the specific differentiation medium, cells were fixed and stained by the von Kossa method (for calcium deposition), with Sudan II and Scarlet stains (for fat accumulation), or immunostained with anti-type II collagen. Cells were analyzed with a model Axioskop 2.0 Zeiss microscope (Germany) equipped with an Axiocam camera (Zeiss).

Transmission electron microscopy

Cells were cultured for 48 hours, then rinsed twice with phosphate-buffered saline, fixed in 2% glutaraldehyde (EM Sciences, Fort Washington, PA, USA), 2% paraformaldehyde (EM Sciences) in 0.1 M cacodylate (EM Sciences) buffer (pH 7.4), containing 0.05% $CaCl_2$, postfixed in 1% OsO_4 (EM Sciences), and embedded in Embed 812 (EM Sciences). Thin sections were stained for 10 minutes with Reynolds lead citrate and uranyl acetate [44], and examined in a Phillips 208 Electron Microscope.

Flow cytometry

The immunophenotypic characterization was performed by flow cytometry using the following 22 monoclonal antibodies: CD-33-fluorescein isothiocyanate (FITC), CD45-FITC, CD31-FITC, HLA-DR-FITC, cadherin-5-FITC, glycophorin-FITC, CD73-phycoerythrin (PE), CD146-PE, CD90-PE, CD29-PE, CD44-PE, CD13-PE, CD49e-PE, HLA-ABC-PE, CD34-PE, CD14-PE, CD54-PE, CD166, and AC-133-PE (Pharmingen, San Jose, CA, USA), STRO-1 (kindly provided by B. Torok-Storb, Fred Hutchinson Cancer Research Center, Seattle, WA, USA), FSP-1 (Dako, Glostrup, Denmark) and KDR (vascular endothelial growth factor receptor-2; Sigma-Aldrich). Cells were incubated with the antibodies following manufacturer's instructions. For FSP-1 intracellular detection, cells were fixed with 1% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with antibody against FSP-1. Nonspecific IgG of the corresponding class served as the negative control. Cell suspensions were analyzed on a FACSort flow cytometer (Becton-Dickinson, Mountain View, CA, USA) using CellQuest software.

RNA extraction

After isolation, cells were centrifuged and resuspended in phosphate-buffered saline treated with 0.1% diethylpyrocarbonate, total RNA was obtained using the TRIzol-LS reagent (cat. no. 10296010, Invitrogen Corp., Carlsbad, CA, USA) and stored at –70°C. RNA was quantified and the quality checked by the integrity of the 28S and 18S bands on 1% agarose gel and by RT-PCR. RNA from other human tissues was obtained by maceration in Trizol-LS using a Polytron homogenizer.

SAGE transcriptome

Total RNA of cultured retinal perivascular cells from adult retina was used to generate the SAGE library. Library construction and data analysis were done as described previously for BM and UCV MSC [37] using the I-SAGE Kit (cat. no. T5001-01, Invitrogen Corp.).

Semi-quantitative RT-PCR

In addition, to validate SAGE results semi-quantitative RT-PCR of a set of 39 genes was performed in more extensive cell types. These samples corresponding to 14 cultures of plastic-adherent multipotent MSCs isolated from different sources (5 from adult tissues, 8 from fetal tissues, and 1 from adult BM transfected with a plasmid expressing the green fluorescent protein); 5 fibroblast cultures; 2 MSC cultures from BM differentiated in vitro into adipocytes or osteoblasts; 2 samples of pericytes obtained by different approaches; and 1 sample each of total BM, HUVEC, liver, brain, skeletal muscle, skin, and heart. The analyzed gene set includes 24 genes involved in regulation of mesenchymal-derived cells, such as, vimentin; S100A4; extracellular matrix components (LGAL-S; BGN, COL1A1, COL1A2); components of transforming growth factor-β (activin subunit A [INHBA]) and mitogen-activated protein kinase (early growth response 1 [EGR1]) and angiogenesis (matrix metalloprotease-2 and interleukin-8) signaling pathways; regulators of cell-cycle machinery (cyclin B1 [CCNB1] and CHK1 check point homolog [CHEK1]); and others. Also, 15 unrelated genes, which include genes from: 1) cardiac muscle differentiation (transcription factor GATA4); 2) adipogenic differentiation (insulin growth factor binding protein 2 [IGFBP2]); 3) blood coagulation (FGG); 4) neural cells (NCAM2 and NMU); 5) adherence junction of epithelial cells (Keratin 5), as well as other genes analyzed. Total RNA isolated from samples was reverse transcribed to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA; www.appliedbiosystems.com), following manufacturer's instructions. PCR was initially carried out in a total volume of 25 μL, using cDNA equivalent to 20 ng initial RNA; when the reaction was positive for undiluted samples, the cDNA was serially diluted (1:2 to 1:64) before being used in PCR. Primer sequences for the evaluated genes are listed in a Supplemental Table (Table S1; www.hemocentro.fmrp.usp.br/covas2007supplfile.htm).

Real-time PCR

We assessed expression of CD140B, NG2, FSP-1, CD271, CD146, CD31, and vascular endothelial–cadherin by quantitative real-time PCR in order to compare multipotent MSCs, retinal perivascular

cells isolated by plastic adherence properties, purified pericytes isolated by CD146 immunoselection, fibroblasts, and HUVEC. Moreover, we evaluated the expression levels of CD146 and FSP-1 on a set of 16 samples, including both retinal perivascular cells, 11 MSC and 3 fibroblasts. Real-time quantitative PCR was performed on an ABI Prism 7500 sequence detection system (Applied Biosystems) using SYBR green chemistry and TaqMan PCR assay. Gene expression was normalized relative to the endogenous gene glyceraldehyde-3-phosphate dehydrogenase and their relative expression in the different cell types were obtained by the Pfaffl method [45]. Probes and primer sequences for the evaluated genes are listed in Supplemental Table 2.

Clustering

In addition to the retinal perivascular cell library and four other libraries generated by us, we also used libraries available at the Gene Expression Omnibus site (http://www.ncbi.nlm.nih.gov/geo/), or available as supplemental data or upon request to authors, as listed in Table 1. After normalization of the total tag counts to 200,000 tags in all libraries, the clustering of SAGE data was carried using three distinct tag sets corresponding to the top 100, 200, or 500 most-expressed tags of the libraries used. After excluding the redundancy, these sets corresponded, respectively, to 428, 821, or 2293 unique tags, which were used to cluster the libraries by the average linkage method using Spearman's rank correlation as the similarity metric, with the software Cluster 3.0 and Treeview [46,47].

In order to control the clustering procedure, and to take into account the effect of experimental variations on the similarities of cell profiles, we used three sets of normal tissues or cells (muscle, endothelial, and hematopoietic), each set composed of two similar libraries from the same study and a third library of a similar tissue or cell from a distinct study (Table 1).

Results

Morphological and functional characterization of plastic-adherent multipotent MSC, fibroblasts and CD146$^+$ sorted or unsorted perivascular cells derived from retina

We compared morphologically and functionally BM MSC with plastic-adherent multipotent MSCs from six fetal tissues (carotid, muscle fascia, testis, liver, lung, and thymus) and seven adult tissues (umbilical cord blood, umbilical vein, umbilical artery, BM, saphena vein, adipose tissue, and retina). Also, we compared it to fibroblasts from three sources (abdominal skin, muscle fascia, and foreskin), commercial fibroblast and sorted CD146$^+$ perivascular retinal cells. Morphological analysis showed that all cell populations showed a considerable morphological similarity. Figure 1A, B, E, and F is a representative picture of two of these cultures (BM MSC and fibroblast). Moreover, we performed the ultrastructural analysis of these two cell types. As shown in Figure 1C, D, G, and H, these cells present a large nuclei with prominent nucleoli, abundant rough endoplasmic reticulum, and numerous mitochondria, which is characteristic of metabolically active cell.

The light microscopy analysis demonstrated that these cells maintained a stable morphology for about 15 to 20 passages in culture without spontaneous differentiation. After that, the cells became more rectangular, with a decrease of the nucleus-to-cytoplasm ratio that was interpreted as a sign of senescence.

In order to further characterize the differentiation capacity, we cultured these cell types under adipogenic, osteogenic, and chondrogenic conditions. We found that plastic-adherent MSC cells derived from several fetal and adult tissues, as well as from retinal perivascular cells, fibroblasts and highly purified retinal CD146$^+$ cells showed a homogeneous capacity for differentiation into adipocytes, chondrocytes, and osteocytes, although plastic adherent MSCs derived from adipose tissue did not differentiate in osteoblasts. The culture of commercial fetal fibroblast behaved similarly, whereas foreskin fibroblasts did not differentiate (Table 2), which may be related to culture age-related changes due to the large number of passages. Figure 1I to Q shows a representative picture of BM MSC, fibroblast, and plastic-adherent perivascular cells derived from retina after differentiation induction in three mesodermal lineages.

Immunophenotypic profile

Flow cytometry analysis of all plastic adherent cells isolated from the various tissues and fibroblast were positive for the CD73, CD90, CD29, CD13, CD44, CD49e, STRO-1, and HLA-class I markers, and negative for markers of hematopoietic cells (CD34, CD14, CD45, glycophorin A), endothelial cells (CD31, cadherin 5, KDR) and HLA-class II (Table 3). A distinct pattern was noted for FSP-1 and CD146 between fibroblast and plastic-adherent MSC cells: fibroblasts showed a low percentage of CD146-positive cells and high percentage of FSP-1 positive cells, whereas most of the plastic-adherent MSC cells showed the inverse.

Regarding perivascular cells derived from retina, we analyzed this panel of cell surface markers for the unsorted and sorted (CD146$^+$) cell populations. We observed that they showed a similar level of expression for most cell surface markers. However, CD146 and CD166 were slightly increased, when compared to the heterogeneous population.

Also, as expected, HUVEC exhibited a different profile because they were positive for CD31 and cadherin 5. Also, they were negative for hematopoietic cell markers, but they shared some cell surface markers with plastic-adherent multipotent mesenchymal cells (Table 3).

Pericyte transcriptome

A total of 33,513 tags were obtained by sequencing. Excluding redundancy, these results correspond to 10,663 unique tags, of which 7782 matched known genes or expressed sequence tags in the CGAP SAGE Genie mapping (6446 unique UniGene clusters), whereas 2881 unique tags had no matches. The complete list of tags found in retinal

Table 1. Human serial analysis of gene-expression libraries used for cluster analysis

Tissue	Abbreviation	Total tags	Unique tags	Reference or GEO Acc. No.
CTC MSC from BM	MSC-BM_CTC	102,796	34,649	[38]
CTC MSC from UCV	MSC-UCV_CTC	100,922	29,407	[37]
CTC retinal perivascular cells	RPC_CTC	33,513	10,663	Present study
CTC CD34 purified cells from BM	CD34-BM_CTC	61,302	24,274	[83]
CTC CD34 purified cells from UCB	CD34-UCB_CTC	60,745	22,094	[83]
CD34 purified cells from BM	CD34-BM	99,954	42,399	[84]
HUVEC	HUVEC	37,048	13,258	[85]
Microvascular endothelial cells	Vasc-Ctr	51,642	20,562	GSM706
Microvascular endothelial cells + VEGF	Vasc-VEGF	57,316	21,232	GSM707
BJ dermal fibroblasts	Fibroblasts	57,573	21,839	[86]
Smooth muscle cells	Smooth muscle	51,398	17,268	[87]
Myofibroblasts derived from stellate cells	Stellate-Myofibr	13,072	6,306	[34]
Hepatic stellate cells at day 0	Stellate-0	15,360	7,776	[34]
Hepatic stellate cells at day 15	Stellate-15	27,676	10,189	[34]
Heart myocytes	Heart	84,357	20,445	GSM1499
Striate muscle from old individual	Muscle-old	53,853	13,835	[88]
Striate muscle from young individual	Muscle-young	53,875	12,207	[88]

The first five libraries labeled CTC were obtained in our laboratory.
BM = bone marrow; HUVEC = human umbilical vein endothelial cells; MSC = mesenchymal stromal cells; UCB = umbilical cord blood; UCV = umbilical cord vein; VEGF = vascular endothelial growth factor.

perivascular cells (RPC) can be found at our Web site http://www.hemocentro.fmrp.usp.br/covas2007supplfile.htm. The top 85 tags found in retinal perivascular cells and the corresponding counts in BM and UCV MSC are shown in Table 4.

Real-time PCR evaluation of selected transcripts

Transcripts of NG2 and CD146 are highly expressed in $CD146^+$ pericytes and lower expressed in BM-MSCs, plastic-adherent RPCs and fibroblasts. Moreover, transcripts of FSP-1 are highly expressed in skin fibroblast and lower expressed in the other cell types. In addition, increased amounts of CD140b and CD271 mRNAs were found on BM MSC, skin fibroblasts, and sorted $CD146^+$ perivascular cells isolated from retina compared to plastic-adherent RPC. On the other hand, CD31 and vascular endothelial–cadherin are highly expressed in HUVEC and absent on either BM MSCs, plastic adherent RPC, $CD146^+$ pericytes, and fibroblast (Fig. 2A–G).

Inverse correlation between FSP-1 and CD146, observed by immunophenotyping, was also observed by real-time PCR. Analysis of 16 samples revealed a significant inverse correlation (Spearman $r = -0.652$ and p value $= 0.0031$). The fibroblasts had the highest expression levels of FSP-1 and the lowest expression of CD146, while the opposite was observed for pericytes (Fig. 2H).

Similarities between multipotent MSCs, retinal perivascular cells, hepatic stellate cells, and related cell types

Clustering of SAGE data showed that MSC from BM and from umbilical vein were grouped with RPC in all clusters obtained by our approach; Figure 3A is a representative dendrogram (100 top expressed tags, Spearman's rank coefficient). This group also formed a cluster with the stellate hepatic cells in five of the six cluster results (the exception being the top 100 tags clustered by Pearson's correlation coefficient). A grouping of fibroblasts, SMCs, and myofibroblasts (differentiated from stellate cells) was also observed in five of six cluster results. Control sets, which include $CD34^+$ cells, HUVEC, vascular cells, and heart and muscle cells showed the expected clustering (Fig. 3A).

Validation of SAGE data using semi-quantitative PCR

Results of semi-quantitative RT-PCR for 39 selected genes were used to group the cells and tissues on the basis of gene-expression similarities (Fig. 3B). Despite the heterogeneous expression of some genes, MSC, RPC, $CD146^+$ pericytes and fibroblasts shared a common expression pattern that is clearly distinguished from the tissues used for comparison (cardiac and skeletal muscles, liver, brain, skin, and BM), supporting the conclusion that MSCs from fetal and adult tissues, fibroblasts, pericytes, and differentiated MSC share a common pattern of expression of the selected genes.

Discussion

Since the first report [18], experimental evidence that multipotential stem cells are present in the mural wall of the vasculature was perceived [16,17]. Nevertheless, despite their similarities, the relationship among MSC, pericytes, fibroblasts, myofibroblasts, SMC, and other differentially named cells, such as hepatic stellate cells, is poorly characterized.

We have compared MSC from many sources with retinal pericytes using morphological, functional, and gene-expression analysis, in order to test the hypothesis that

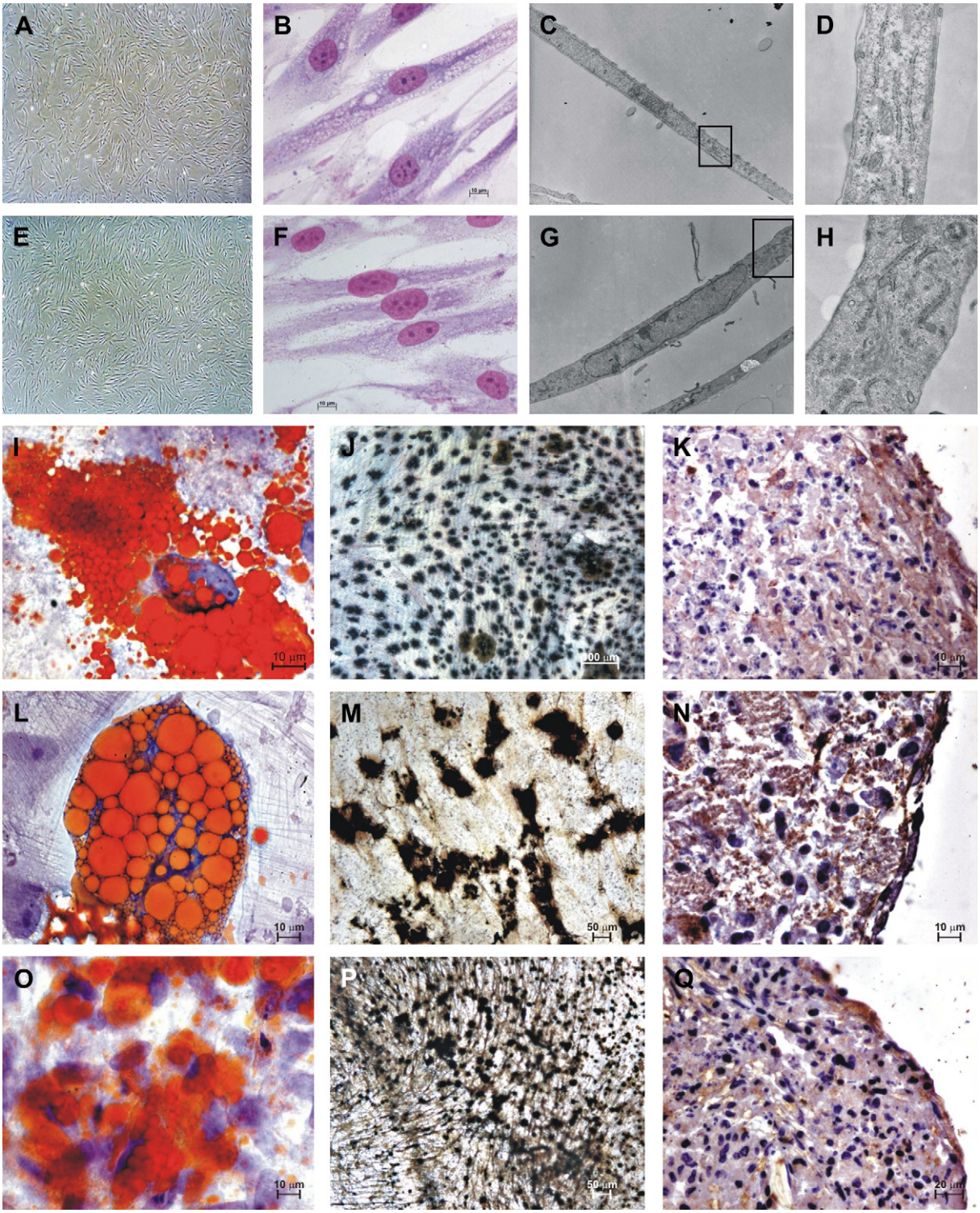

Figure 1. Morphology of human bone marrow-derived mesenchymal stromal cells (MSC) (**A–D**) and human foreskin-derived fibroblast (**E–H**). Phase contrast microscopy (**A**, **E**; magnification ×40); Leishman staining (**B**, **F**); ultrastructure showing the nucleus with a spindle-shape fibroblastic morphology (**C**, **G**); higher magnification of the perinuclear region, showing the rough endoplasmic reticulum (**D**, **H**). Differentiation capacity of bone marrow MSC (**I–K**), pericytes (**L–N**) and skin fibroblast (**O–Q**) into adipocyte (**I**,**L**,**O**) (stained with Sudan II and scarlet), osteocytes (**J**,**M**,**P**) (von Kossa staining), and chondrocytes (**K**,**N**,**Q**) (immunohistochemical demonstration of type II collagen).

Table 2. Differentiation capacity of the diverse cell types isolated from fetal and adult tissues

Cell type	Differentiation		
	Adipocytic	Osteocytic	Chondrocytic
Fetal			
Carotid MSC	+	+	+
Muscle fascia MSC	+	+	+
Testis MSC	+	+	+
Skin MSC	+	+	+
Liver MSC	+	+	+
Umbilical cord blood MSC	+	+	+
Umbilical vein MSC	+	+	+
Umbilical artery MSC	+	+	+
Lung MSC	+	+	ND
Thymus MSC	+	+	+
Fibroblast cell line CCD27Sk	+	+	+
Retinal pericytes	+	+	ND
Adult			
Bone marrow MSC	+	+	+
Saphena vein MSC	+	+	+
Adipose tissue MSC	+	–	ND
Abdominal skin fibroblast	+	+	+
Foreskin fibroblast	–	–	–
Retinal pericytes	+	+	+

MSC = mesenchymal stromal cell; ND = not done.

these cells, as defined by culture methods, are similar and universally distributed in the body. MSC were isolated from 12 different fetal and adult tissues, and compared with retinal pericytes, fibroblasts, and five nonrelated somatic cell types. MSC were isolated from carotid artery, muscle fascia, and testis of the fetus for the first time.

Isolated MSC were homogeneous with respect to morphology, surface markers, and potential to differentiate into osteocytes, adipocytes, and chondrocytes, although MSC derived from adipose tissue differentiated into adipocytes but not osteocytes. In addition, retinal pericytes obtained by two different approaches and fibroblasts derived from four different sources showed morphologic appearance and immunophenotype profiles similar to MSC. Moreover, pericytes and three of four fibroblast cultures could be induced to differentiate. Although cell source may influence the differentiation potential [48], the inability of the foreskin fibroblast tested to differentiate may be related to culture age-related changes due to the large number of passages. Thus, on the basis of morphologic, functional, and immunophenotypic characteristics, MSC isolated from various sources, retinal pericytes and fibroblasts are similar in relation to immunophenotype [49] and differentiation capacity of MSC [1].

The gene-expression profile of retinal pericytes is also very similar to the one related to MSC. Our SAGE clustering results show a consistent grouping of MSC, pericytes, and hepatic stellate cells, independent of the set of tags used, which indicates the close relationship of these cells. This result was further validated by consistently grouping the control tissues (vascular, muscle, and hematopoietic) as expected. In addition, clustering of fibroblasts, SMCs, and myofibroblasts differentiated from hepatic stellate cells indicates that these cells are also closely related. The separated clustering of these groups would reflect differences related to the differentiation state of MSC, pericytes, and hepatic stellate cells, compared to fibroblasts, SMCs, and myofibroblasts differentiated from stellate cells.

The inter-relationships implied by these results are in agreement with data showing functional relationship between MSC, fibroblasts, and myofibroblasts [27,50,51], and their involvement in the vessel function as well as in pathologic processes, such as pulmonary, hepatic, and renal fibrosis [31–33]. In an experimental model of liver cirrhosis, 70% of the myofibroblasts and 68% of the hepatic stellate cells (which are similar to the pericytes in the liver) found in the cirrhotic liver derived from the BM, implicating the BM MSCs as the major origin of the effectors of the fibrotic process, the hepatic stellate cells and myofibroblasts [35].

Furthermore, the large overall similarity between pericytes and MSC is in line with published data showing similar immunophenotype [25,52,53] and differentiation potential in vitro [19,21,22,54,55] and in vivo [56].

The common expression of many transcripts identified by SAGE were further validated by semi-quantitative RT-PCR, comparing the gene expression of a set of selected genes for MSCs derived from several tissues, fibroblasts, and retinal pericytes. For instance, genes such as VIM, LGALS1, ANXA2, MMP2, TAGLN, TAGLN2, SPARC, and others (Fig. 3B), are expressed at similar levels among those cells. This indicates a close proximity in biological terms, in agreement with the functional and morphological studies.

Despite this overall similarity, there was a heterogeneous expression of genes such as INHBA, EGR1, interleukin-8, and CXCL6 when comparing the cell cultures obtained from distinct sources. MSC derived from UCV, adult vein, artery, pericytes, and fibroblasts in general share a higher expression of CXCL6 or interleukin-8, which are factors related to angiogenesis [57]. This may indicate the conservation of locally acquired source-related characteristics, in agreement with our previous work [37] and with the heterogeneity found for fibroblasts derived from different sources [58–60].

Although MSC and pericytes share with fibroblasts a global gene-expression pattern, some of the genes evaluated displayed important differences between these cells. For example, periostin and biglycan were highly expressed in pericytes and in the majority of the MSC cultures, but they were expressed at lower levels in three and four, respectively, of the five fibroblasts tested. Because the targeted disruption of these genes causes abnormal cartilage and bone formation [61,62], their low levels on fibroblasts may be

Table 3. Immunophenotypes of mesenchymal stromal cells, human umbilical vein endothelial cells, pericytes, and fibroblasts isolated from fetal and adult tissues (percentage of positive cells on flow cytometry)

	Fetal MSC						Adult MSC						Pericytes			Fibroblasts			
Marker	Ca	MF	Te	Liv	Lun	Thy	UCB	UV	UA	BM	SV	AT	AD	CD146	HUVEC	CCD27	Skin	MF	Fsk
CD73	62	68	68	48	80	84	ND	96	85	82	65	97	77	64	42	66	93	95	93
Stro1	42	33	26	11	ND	34	ND	18	7	20	26	19	16	14	8	ND	7	3	46
CD90	97	99	97	96	98	98	96	98	99	97	96	99	91	92	4	98	99	96	99
CD29	90	92	93	96	97	95	95	98	97	89	97	96	78	94	98	84	99	91	98
CD13	65	95	68	80	87	93	91	94	91	94	94	96	90	76	99	95	99	62	99
HLAI	51	65	84	49	57	65	90	95	55	78	94	46	88	82	25	41	94	73	77
CD49e	77	89	83	82	92	93	ND	35	94	89	91	93	42	85	99	53	99	90	97
CD54	46	41	89	74	62	49	55	73	55	55	67	34	30	38	68	18	68	70	94
CD44	52	56	41	66	87	69	59	86	83	87	88	60	89	87	86	62	91	50	83
CD34	3	0	0	0	0	3	1	1	0	0	5	3	0	0	3	4	11	1	6
CD45	0	0	0	0	0	0	0	0	0	0	0	0	11	0	1	0	0	0	0
CD14	1	0	0	5	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0
HLADR	1	0	1	0	0	1	1	2	1	2	1	1	1	1	0	4	3	2	0
CD33	1	0	1	0	0	0	ND	0	0	1	0	0	0	2	0	2	1	1	1
KDR	4	2	3	0	6	1	2	2	4	1	1	1	3	3	11	7	3	2	5
GLYPA	1	2	9	2	2	3	ND	1	1	4	1	ND	0	5	1	0	2	1	1
AC 133	0	0	2	0	0	0	ND	1	0	1	1	ND	0	ND	2	1	2	1	2
CD146	43	41	65	ND	29	41	ND	31	ND	73	ND	ND	66	83	37	8	2	9	9
FSP-1	4	40	10	ND	24	7	ND	15	0	11	4	ND	ND	ND	8	84	90	ND	28
CD166	58	87	76	12	48	52	ND	15	46	69	63	ND	59	71	73	34	68	79	46
CADH5	0	1	3	0	3	1	ND	2	4	1	3	2	0	3	59	9	3	ND	0
CD31	0	0	1	0	1	1	ND	1	1	1	0	1	1	1	86	1	4	0	0

All the results are means of two to six measurements (see text, Results).
AD = obtained by adhesion; Amn = Amnion; AT = adipose tissue; BM = bone marrow; Ca = carotid; CD146 = obtained by sorting with anti-CD146; CCD27 = commercial cell line; Fsk = foreskin; HUVEC = human umbilical vein endothelial cells; Liv = liver; Lun = lung; MF = muscle fascia; MSC = mesenchymal stromal cells; ND = not done; pericytes = retinal pericytes; SV = saphena vein; Te = testis; Thy = thymus; UA = umbilical artery; UCB = umbilical cord blood; UV = umbilical vein.

related to reduced differentiation potential or a distinct functional commitment.

Moreover, the inverse correlation found for transcript levels of FSP-1 (S100A4) and CD146 on the samples evaluated, places fibroblast and pericytes at opposite ends, while MSC showed variable but inversely correlated levels of these two transcripts. The immunophenotyping of selected cell cultures with anti-FSP1 roughly corresponded to these findings: two of the fibroblast cultures showed the greatest percentage of positive cells, whereas MSC showed a variable but lower percentage of positive cells, which corroborates the higher expression found by real-time PCR. These findings suggests that transcripts for the FSP-1 gene would be enriched in the CD146-negative fraction of the cells. This suggestion is supported by the finding that fibroblasts had $<10\%$ of the cells positive for CD146.

Also, FSP-1 is considered a fibroblast marker [63] and it is not found in any cell from the mouse embryo until day E9, or in early mesenchymal cells, but appears in adult tissue fibroblasts and during tissue fibrosis [63,64]. The CD146 (S-Endo 1) is a marker present in pericytes and endothelial, smooth muscle, stromal, and perivascular mesenchymal stem cells [25,65–67], involved in cell-to-cell junction [68] and in an outside-in signaling pathway comprising proteins, such as the tyrosine kinases FYN, FAK, and paxillin [69]. Given that the activation of FAK and paxillin is involved in the osteogenic differentiation of MSC, mediated by interaction with extracellular matrix components, such as vitronectin or laminin-5 [70–72], it is reasonable to propose that CD146 could be used as a marker for more primitive mesenchymal stem cells, a proposition also supported by the work of Baksh et al. [73].

CD146 is considered a marker of circulating endothelial progenitors, too [74]. Accordingly, HUVECs showed a considerably high proportion of CD146-positive cells (37%), but instead, this differed from all MSC and pericytes in that 86% of the cells in culture were also positive for the endothelial marker CD31. Moreover, transcripts of CD31 and vascular endothelial–cadherin, evaluated by real-time PCR, are absent on either BM MSCs and $CD146^+$ pericytes and highly expressed in HUVEC. Finally, $CD146^+$ cells isolated from retina displayed increased transcript amounts of typical pericyte markers, such as CD146, NG2, and CD271, compared to plastic-adherent isolated cells from the retina and absent in HUVEC. Together, these data demonstrate that CD146-selected cells from retina represent a pericyte-enriched population differing from $CD146^+$ HUVEC.

Thus, our data and the previous reported evidence indicate that MSC and pericytes are related cells located

Table 4. Top 50 expressed tags from the pericyte serial analysis of gene-expression library remaining from the top 85 more expressed tags after excluding 35 tags corresponding to ribosomal proteins

TAG	Symbol	Description	Perivascular retinal adherent cells	MSC BM	MSC UCV	Fibroblast GSM14916
CCCATCGTCC		Transcribed locus, weakly similar to XP_395705.3 similar to CG18497-PA, isoform A, partial [Apis mellifera]	3443	1377	1132	549
TTGGGGTTTC	FTH1	Ferritin, heavy polypeptide 1	3336	650	648	427
TACCATCAAT	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	2178	1206	1359	962
GAAGCAGGAC	CFL1	Cofilin 1 (nonmuscle)	1570	1222	1365	382
GCCCCCAATA	**LGALS1**	Lectin, galactoside-binding, soluble, 1 (galectin 1)	1426	1121	2434	1053
CCTAGCTGGA	PPIA	Peptidyl prolyl isomerase A (cyclophilin A)	1420	728	1076	368
AGCACCTCCA	EEF2	Eukaryotic translation elongation factor 2	1307	658	725	129
CACCTAATTG			1229	444	321	490
GGCTGGGGGC	PFN1	Profilin 1	1194	625	1027	591
TAGGTTGTCT	TPT1	Tumor protein, translationally controlled 1	1176	732	923	868
TGTGTTGAGA	EEF1A1	Eukaryotic translation elongation factor 1 α1	949	718	444	552
ATGTGAAGAG	**SPARC**	Secreted protein, acidic, cysteine-rich (osteonectin)	943	1498	1639	921
GTGACCACGG	GRIN2C	Glutamate receptor, ionotropic, N-methyl D-aspartate 2C	889	39	67	76
TTCATACACC			871	636	190	629
GTGTGTTTGT	TGFBI	Transforming growth factor, β-induced, 68 kDa	835	1434	949	782
GTGCTGAATG	MYL6	Myosin, light chain 6, alkali, smooth muscle, and nonmuscle	770	374	745	431
TTGGTGAAGG	TMSB4X	Thymosin, β4, X-linked	633	444	834	212
GAAATACAGT	NT5C	5′, 3′-nucleotidase, cytosolic	615	177	125	87
TGTACCTGTA	TUBA1B	Tubulin, α1b	591	656	922	511
GGGGAAATCG	TMSB10	Thymosin, β10	519	379	440	399
GCGCAGAGGT			507	121	230	94
AGCCCTACAA			501	132	121	111
GGTCCAGTGT	PGAM1	Phosphoglycerate mutase 1 (brain)	465	218	291	146
CCCTGGGTTC	FTL	Ferritin, light polypeptide	460	156	117	135
CTAAGACTTC	LOC645221	Hypothetical LOC645221	442	220	59	577
TTGTAATCGT	OAZ1	Ornithine decarboxylase antizyme 1	436	179	424	156
GTGAAACCCC	PAFAH2	Platelet-activating factor acetyl hydrolase 2, 40 kDa	430	230	422	566
TGGGCAAAGC	EEF1G	Eukaryotic translation elongation factor 1γ	424	222	402	132
CTAGCCTCAC	ACTG1	Actin, γ1	424	475	262	292
TGGAAATGAC	**COL1A1**	Collagen, type I, α1	418	2566	1373	417
GTCTGGGGCT	TAGLN2	Transgelin 2	412	220	466	90
GTTGTGGTTA	B2M	β-2-microglobulin	412	426	462	247
TGTGATCAGA	ATP5L	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G	406	198	281	135
CCCCAGTTGC	CAPNS1	Calpain, small subunit 1	364	259	273	236
ACTTTTTCAA		CDNA clone IMAGE:4328048	352	597	81	135
GCAAAAAAAA	PDE6D	Phosphodiesterase 6D, cGMP-specific, rod, δ	346	173	250	212
TTTGCACCTT	CTGF	Connective tissue growth factor	334	523	323	424
CATATCATTA	IGFBP7	Insulin-like growth factor binding protein 7	322	243	81	448
TGCATCTGGT	HSPA5	Heat shock 70-kDa protein 5 (glucose-regulated protein, 78 kDa)	322	214	519	80
GACTCACTTT	PPIB	Peptidyl prolyl isomerase B (cyclophilin B)	304	173	321	163
GGGAAGCAGA	F11R	F11 receptor	304	469	527	201
TGCCTCTGCG	CD151	CD151 molecule (Raph blood group)	298	200	258	184
GGAGTGTGCT	MYL9	Myosin, light chain 9, regulatory	292	449	392	153
GGCCCTGAGC	POLR2L	Polymerase (RNA) II (DNA directed) polypeptide L, 7.6 kDa	286	93	176	42
TTGGAGATCT	NDUFA4	NADH dehydrogenase (ubiquinone) 1α subcomplex, 4, 9 kDa	280	154	200	97
GTAAGTGTAC			280	154	486	181
TTGGCAGCCC		Transcribed locus, strongly similar to XP_001106319.1 similar to ribosomal protein L27a [Macaca mulatta]	275	53	182	83
AGAAAGATGT	ANXA1	Annexin A1	275	136	105	111
TGAGGGAATA	TPI1	Triosephosphate isomerase 1	275	294	289	191
GGATATGTGG	EGR1	Early growth response 1	257	224	101	14

Transcripts in bold were evaluated by semi-quantitative polymerase chain reaction.
MSC BM = mesenchymal stromal cells bone marrow; MSC UCV = mesenchymal stromal cell umbilical vein.

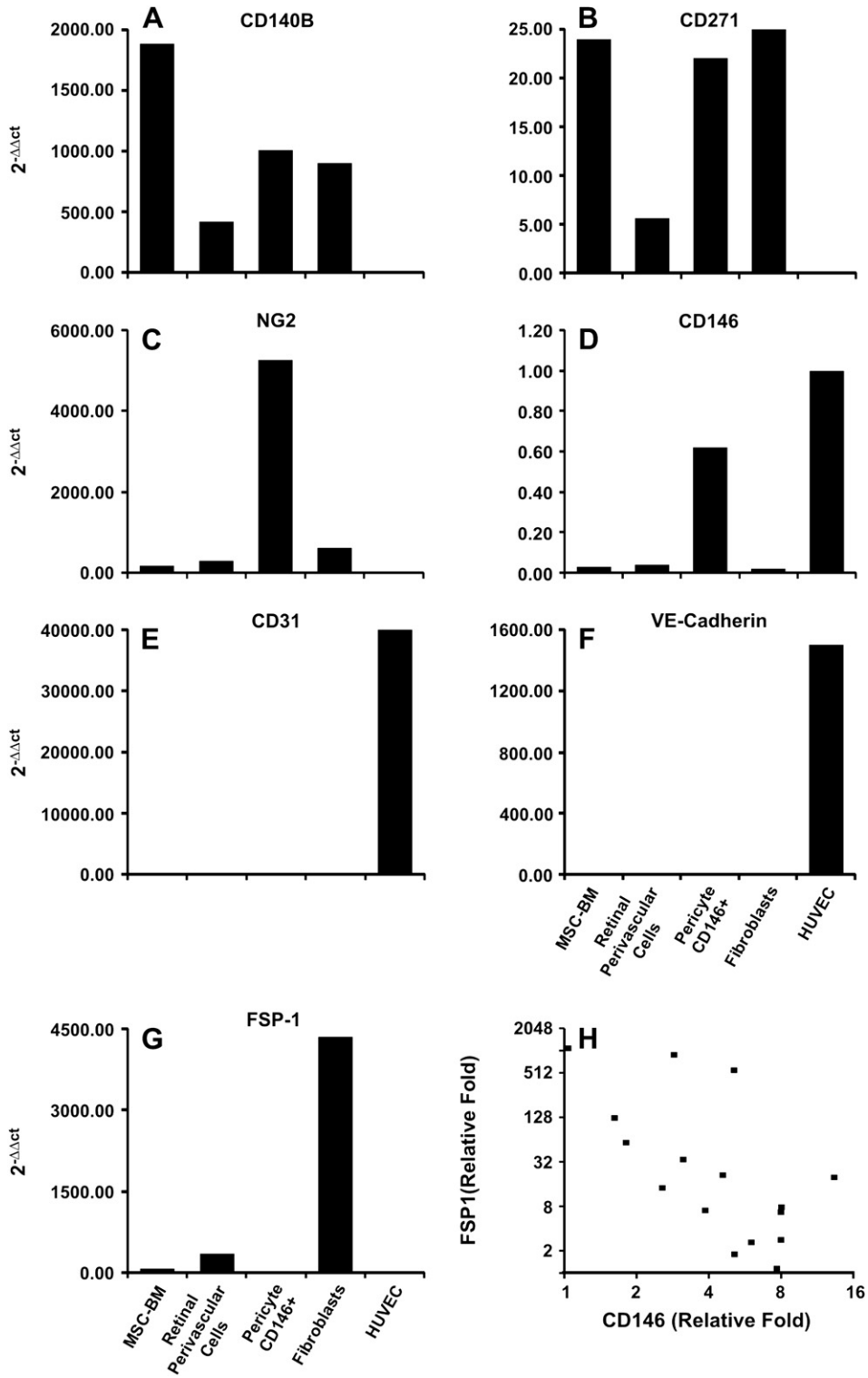

Figure 2. Real-time polymerase chain reaction quantitation of selected transcripts and correlation analysis. (**A**–**G**) Transcript levels of CD140B, CD271, NG2, CD146, CD31, vascular endothelial–cadherin and fibroblast-specific protein-1 (FSP-1) were evaluated on sorted $CD146^{+}$ and plastic-adherent perivascular cells isolated from retina, bone marrow (BM) mesenchymal stromal cells (MSC), skin fibroblasts and human umbilical vein endothelial cells (HUVEC). (**H**) Transcript levels of FSP-1 and CD146 were evaluated on 16 samples, including 11 MSC, 2 pericytes, and 3 fibroblasts. Results are showed as the fold increase, relative to the sample with the lowest transcript level. A nonparametric test (Spearman Rank) was carried, demonstrating a significant (p value = 0.0031) inverse correlation (r = –0.652) between both transcripts.

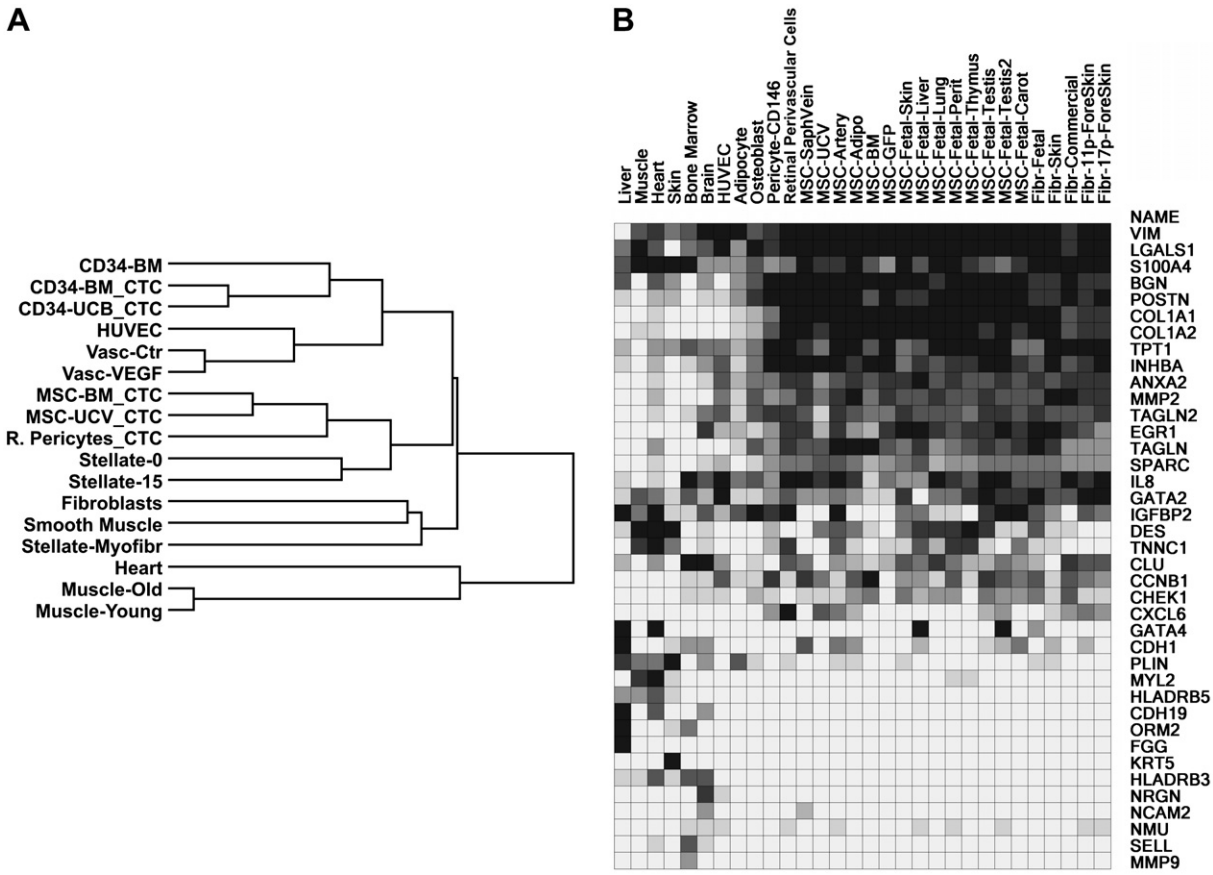

Figure 3. (**A**) Clustering of selected tissues and cells based on serial analysis of gene expression (SAGE) data, Spearman's rank correlation, set of 100 top expressed tags for each tissue (total of 428 tags) (names and sources in Table 1). (**B**) Heatmap of semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) data. Expression level of 39 selected transcripts were evaluated on 30 different samples. The intensity in the gray-scale is proportional to the highest dilution factor in which PCR was positive.

in the subendothelial layer of the vasculature where they function as a source of cells for repair and maintenance of the various tissues. The isolation of MSC from the subendothelium of various arteries and veins, including umbilical vein [36,75,76], saphena vein [39], umbilical artery and fetal carotid (in this article), and bovine aorta [16,17] is in agreement with our hypothesis.

This hypothesis is supported by the recent findings that the more primitive and proliferating MSC from the BM express CD271 (neurotrophin receptor) and that depletion of the neurotrophin receptor prevents dedifferentiation of hepatic stellate cells (also described as hepatic pericyte) [77] into myofibroblasts and regeneration of hepatocytes in a model of liver injury [78,79].

Recently, it has been proposed that a cell continuum may exist from fibroblast to myofibroblast to mature vascular smooth muscle cells in the artery wall [80,81], and that fibroblasts, myofibroblasts, and vascular smooth muscle cells derive from a common stem cell [82]. Our data support this hypothesis and indicate that MSC, fibroblasts, and pericytes would be related cells present at the vascular wall, constituting a MSC compartment extending throughout the entire organism.

In conclusion, we present experimental evidence that human MSC and pericytes, as operationally defined by culture methods, are similar cells located in the wall of the vasculature, where they function as sources of cells for repair and tissue maintenance. This proposition has implications about the way we define those cells and opens new avenues for further studies in basic as well as clinical models.

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