Mesenchymal stem cells reside in virtually all post-natal organs and tissues

Lindolfo da Silva Meirelles, Pedro Cesar Chagastelles and Nance Beyer Nardi*

Departamento de Genética, Universidade Federal do Rio Grande do Sul, Av Bento Gonçalves 9500, 91540-970 Porto Alegre, RS, Brazil

*Author for correspondence (e-mail: nardi@ufrgs.br)

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Summary

Mesenchymal stem cells (MSCs) are multipotent cells which can give rise to mesenchymal and non-mesenchymal tissues in vitro and in vivo. Whereas in vitro properties such as (trans)differentiation capabilities are well known, there is little information regarding natural distribution and biology in the living organism. To investigate the subject further, we generated long-term cultures of cells with mesenchymal stem cell characteristics from different organs and tissues from adult mice. These populations have morphology, immunophenotype and growth properties similar to bone marrow-derived MSCs. The differentiation potential was related to the tissue of origin. The results indicate that (1) cells with mesenchymal stem characteristics can be derived and propagated in vitro from different organs and tissues (brain, spleen, liver, kidney, lung, bone marrow, muscle, thymus, pancreas); (2) MSC long-term cultures can be generated from large blood vessels such as the aorta artery and the vena cava, as well as from small vessels such as those from kidney glomeruli; (3) MSCs are not detected in peripheral blood. Taken together, these results suggest that the distribution of MSCs throughout the post-natal organism is related to their existence in a perivascular niche. These findings have implications for understanding MSC biology, and for clinical and pharmacological purposes.

Key words: Mesenchymal stem cell, Pericyte, CFU-F, Mouse, In vitro cultivation

Introduction

Stem cells are defined as having the capacity for extensive self-renewal and for originating at least one type of highly differentiated descendant (Watt and Hogan, 2000). Post-natal tissues have reservoirs of specific stem cells which contribute to maintenance and regeneration. Examples include epithelial stem cells in epidermis and intestinal crypts (Slack, 2000), neural stem cells in the central nervous system (McKay, 1997) and satellite cells in muscle (Charge and Rudnicki, 2004). The adult bone marrow shelters different types of stem cells, including hematopoietic (Weissman, 2000) and mesenchymal (Prockop, 1997; Nardi and da Silva Meirelles, 2006) stem cells.

Experiments using bone marrow cells have raised the issue of phenotypic plasticity (Herzog et al., 2003; Wagers and Weissman, 2004), because they have shown the consequent generation of specialized cells derived from bone marrow in the central nervous system (Eglitis and Mezey, 1997; Mezey et al., 2000), skeletal muscle (Ferrari et al., 1998), liver (Petersen et al., 1999) and heart (Orlic et al., 2001). The specific cell type(s) involved in these phenomena is not clear. However, reports describing mesenchymal stem cell (MSC) differentiation capabilities suggest that they may contribute to the results observed: they can differentiate into specific cell types in vitro and in vivo (Woodbury et al., 2000; Kopen et al., 1999; Sato et al., 2005), and have a tendency to acquire tissue-specific characteristics when co-cultured with specialized cell types or exposed to tissue extracts in vitro (Houghton et al., 2004; Choi et al., 2005; Lange et al., 2005). In addition, the capacity to differentiate into mesodermal (Pittenger et al., 1999), ectodermal (Kopen et al., 1999) and endodermal (Sato et al., 2005) cell lineages characterizes MSCs as pluripotent cells, suggesting that the term 'mesenchymal' stem cell might be inappropriate to describe this particular stem cell. Since different methodologies are used to cultivate and characterize MSC-related cell types, there is still a lack of consensus on the hierarchy intrinsic to the MSC compartment (Nardi and da Silva Meirelles, 2006), reflected by the existence of similar cells such as multipotent adult progenitor cells (MAPCs) (Reyes et al., 2001), marrow-isolated adult multilineage inducible (MIAMI) cells (D'Ippolito et al., 2004), and recycling stem cells (RS-1, RS-2) (Colter et al., 2000).

The exact nature and localization of MSCs in vivo remain poorly understood; growing evidence indicates a relationship with pericytes (Doherty et al., 1998; Farrington-Rock et al., 2004). Approaches so far include the use of selected markers known to be expressed by MSCs in vitro to seek positive cells in vivo (Bianco et al., 2001; Shi and Gronthos, 2003) and the infusion of marked cultured cells in vivo to analyze their tissue distribution (Anjos-Afonso et al., 2004). The first type of approach, though sensitive, may be nonspecific as the majority of cell markers are specific only in a given context. The second strategy may be less accurate to study MSC natural distribution in vivo because the cells might engraft nonspecifically in different locations. The systematic isolation of MSCs from different organs and tissues and the evaluation of their characteristics could represent an alternative approach. Studies describing the isolation of post-natal mesenchymal stem cells from different sources – e.g. adipose tissue (Zuk et al., 2001),

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tendon (Salingcarnboriboon et al., 2003), periodontal ligament (Seo et al., 2004), synovial membrane (De Bari et al., 2001) and lungs (Sabatini et al., 2005) – can be found in the literature; however, these isolated studies do not allow the consistent visualization of the distribution of MSCs in the post-natal organism.

Here, we analyzed the in vivo distribution of murine post-natal MSCs through the establishment, long-term culture and functional characterization of MSC populations from different tissues and organs. The possibility that MSC cultures were partially or entirely derived from circulating blood was excluded by perfusing the animals intravascularly before organ collection. Moreover, no MSC long-term culture could be established from blood when a controlled protocol to minimize vessel rupture was used. Our data demonstrate that the MSC compartment is more widely distributed than previously thought and we present evidence that MSCs are resident in vessel walls. Variations in immunophenotype and osteogenic or adipogenic differentiation potential according to the site of origin suggest that functional roles are at least partially organ specific. These findings provide insight on the biology of MSCs in vivo, and add new information to be considered when developing clinical protocols involving the MSC compartment.

Results

Establishment and morphological characterization of long-term cultures

The establishment of long-term cultures was highly dependent on the conditions used to set up the primary culture. For instance, the use of DMEM with 10 mM HEPES instead of common buffered saline to dissolve collagenase, and a digestion time limited to up to 1 hour at 37°C proved to be essential for reproducibility (not shown). Other important factors were the elapsed time between euthanasia and tissue processing – the shorter the better, particularly for bone marrow and liver – and the temperature of the culture medium, which should not be lower than room temperature. The amount of medium used during the collagenase digestion step was also important for the establishment of brain-derived cultures, because this organ rapidly acidifies small volumes of medium. Furthermore, when establishing primary cultures from perfused animals, we observed that using DMEM containing 10 mM HEPES and HB-CMF-HBSS (1:1), rather than culture medium or saline alone, seemed to improve the quality of the MSC-like cells in primary culture.

The MSC long-term cultures generated during this study are described in Table 1. Not all cultures, in particular the earlier ones, were generated using the optimized conditions described above. To minimize eventual differences due to different starting conditions, the analyses were mainly focused on population sets established under similar conditions, even though gross differences, as judged by flow cytometric analyses, morphological and functional characteristics, were not observed among long-term cultures generated in any of the conditions (not shown).

Using the optimized conditions mentioned above, cells that morphologically resembled characterized MSCs could be seen as early as 24 hours post-plating (Fig. 1A). In the case of glomerular cell culture, adherent cell outgrowths could be observed from the third or fourth day onwards (Fig. 1B). Depending on the starting amount and on which tissue was used to establish the cultures, confluence could be reached within 5 days. Individual glomerular cultures did not reach confluence even when cultured for over a month despite robust initial cell growth (Fig. 1B,C), possibly because of the contact inhibition among the cells in each colony after some cell divisions; on the other hand, cultures containing 20 glomeruli or more per well could become confluent within three weeks or so, if the outgrowths were evenly distributed on the bottom of the dish. During the initial culture period (passages 1 to 5) there was some morphological heterogeneity in the adherent fraction, particularly in bone marrow and spleen cultures, possibly because of the presence of hematopoietic contaminants (Fig. 1D). As the cultures were passaged, morphological homogeneity was gradually achieved in that flat cells bearing a large nucleoli-rich nucleus predominated; this was independent of the origin of the culture (Fig. 1E,F). Glomeruli-derived explants, on the other hand, showed this morphology right from the start of culture (Fig. 1B). The expression of surface markers was analyzed by flow cytometry preferably at this time (see below).

During the establishment of primary cultures, special care was taken to avoid contamination by adjacent tissues. Muscle was thoroughly removed from femora and tibiae before bone

Table 1. MSC populations generated and donor animals

Animal	Strain	Age (weeks)	Gender	Perfused	MSC populations generated
001B	BALB/c	14	Male	No	001Bbm
002B	BALB/c	15	Male	No	002Bs
004B	BALB/c	8	Female	No	004Bbm, 004Bs, 004Bt, 004Bk
005B	BALB/c	9	Male	No	005Bs
006B	BALB/c	11	Male	No	006Bb
009B	BALB/c	16	Female	No	009Bl
010B	BALB/c	16	Female	No	010Bb
011B	BALB/c	10	Male	No	011Bt
012B	BALB/c	37	Male	No	012Ba
013B	BALB/c	37	Male	No	013Ba1, 013Ba2
014B	BALB/c	30	Male	No	014Ba1, 014Ba2
015B	BALB/c	31	Male	No	015Ba1, 015Ba2, 015Bs, 015Bl
016B	BALB/c	30	Male	Yes	016Ba1, 016Ba2, 016Bs, 016Bm, 016Bk
017B	BALB/c	43	Male	Yes	017Bk
018B	BALB/c	18	Female	Yes	018Ba1, 018Ba2, 018Bs, 018Bm, 018Bk, 018Bl
019B	BALB/c	20	Female	Yes	019Bs, 019Bbm, 019Blu, 019Bk
021B	BALB/c	27	Female	No	021Bkg
001C	C57Bl/6	39	Female	No	001Ck
002C	C57Bl/6	9	Male	No	002Cbm
008C	C57Bl/6	35	Female	No	008Clu, 008Cvc
009C	C57Bl/6	39	Female	Yes	009Ck
001R	ROSA26	20	Male	No	001Rlu
003R	ROSA26	13	Male	No	003Rbm, 003Rp
001G	GFP	22	Male	No	001Gvc

In the right column, lowercase letters indicate the tissue or organ from which the culture was derived: a, aorta; b, brain; bm, bone marrow; k, kidney; kg, kidney glomeruli; l, liver; lu, lungs; m, muscle; p, pancreas; s, spleen; t, thymus; vc, vena cava. In the case of aorta-derived cultures, the numbers after the 'a' indicate the first and second fraction-derived populations (see Materials and Methods for details).

marrow extraction, to avoid contamination with muscle-derived cells; adipose tissue was likewise separated from the aorta through serial enzymatic dissociation. In this particular case, the two fractions obtained could generate long-term cultures exhibiting the same morphological, immunophenotypic and kinetic characteristics (not shown), indicating that the enzymatic fractionation procedure is not necessary. On the other hand, contamination from surrounding tissue proved to be a serious issue for blood. The establishment of MSC-like long-term cultures from blood collected through cardiac puncture, or from the thoracic cavity, was possible but not easily reproducible (not shown). However, attempts to establish MSC cultures from blood collected from the portal vein were consistently unsuccessful. We consider this a very important observation, because of two facts: first, MSC long-term cultures can be generated from only one cell (see the Cloning section below); second, it is possible that a few MSCs detach from the walls of ruptured vessels and are collected with the blood. In this case, the results would suggest that blood can originate MSC long-term cultures. The insertion of an intravenous catheter cranially into the portal vein helps minimize this problem. As the catheter is introduced contrary to the blood flow, the few cells that eventually detach from the vessel wall during the needle insertion are likely to be lost in the circulation before blood collection starts.

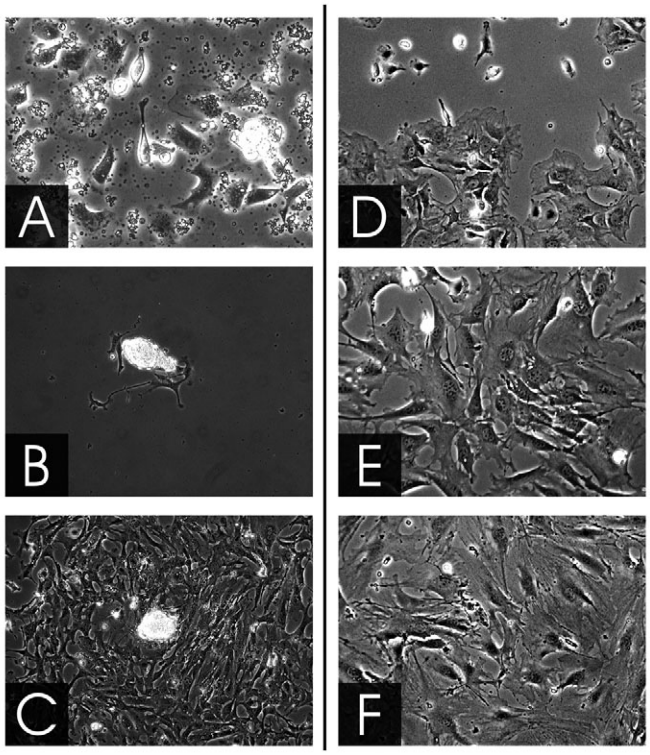

Fig. 1. Morphology of MSC cultures derived from different organs and tissues. (A) Phase-contrast micrographs of MSC-like cells in primary culture of aorta 24 hours after plating. Glomerulus outgrowth on the fourth (B) and sixth (C) day post-plating. (D) Heterogeneity among bone marrow-derived cells at passage 4, with MSC-like cells (lower portion), spindle-shaped and round cells. (E) Pancreas-derived MSCs at passage 30. (F) Vena-cava-derived MSCs at passage 22. Magnifications, ×100 (B-F); ×200 (A).

Immunophenotyping

The analysis of surface markers indicated that the MSC populations originating from multiple sources have a very similar immunophenotype. Examples of surface molecule profiles for selected markers are shown in Fig. 2. All the populations studied expressed CD29 (integrin β_1 chain) and CD44 (hyaluronan receptor). The expression of molecules such as CD34 (hematopoietic progenitor marker), Sca-1 (stem cell antigen-1) and CD49e (integrin α_5 chain) was variable among the different populations, and could also show variation during extended subculture (Fig. 3). CD90.2 expression, when present, was high; however, the proportion of positive cells was variable, ranging from ~30% to nearly 100%. High expression of CD117, the stem cell factor receptor, was infrequent; some populations however seemed to express it at very low levels, and a small percentage of positive cells could sometimes be detected. CD117 expression showed a tendency to decrease during extended serial passage, reaching control levels (Fig. 3), as reported previously for bone-marrow-derived murine MSCs (da Silva Meirelles and Nardi, 2003). The granulocyte marker Gr-1 was expressed in low levels by some populations (not shown). The hematopoietic markers CD45 and CD11b were not expressed by MSCs. They were only observed during the initial passages in cultures derived from bone marrow and spleen, presenting a small proportion of cells with morphological characteristics of macrophages, indicating hematopoietic contamination. MSC cell populations were also negative for the monocyte marker CD13, the leukocyte markers CD18 and CD19, the endothelial marker CD31 and surface Ig. The expression of CD49d (integrin α_4 chain) remains to be analyzed in more detail. When some populations were collected using either trypsin-EDTA or EDTA alone, CD49d was observed on cells collected with EDTA but not on those treated with trypsin, indicating its sensitivity to the enzyme (not shown).

The immunophenotyping of MSCs at early stages revealed the heterogeneity within cell populations before an eventual subpopulation selection owing to extensive cultivation. The expression of αSMA, a vascular smooth muscle cell marker, was analyzed in cultures that had been tested for their differentiation potential. All the MSC populations examined expressed αSMA as exemplified by representative results in Fig. 4, suggesting their relationship to perivascular cells.

Growth kinetics

Growth curves describing culture kinetics were generated as previously described (da Silva Meirelles and Nardi, 2003). Representative examples of growth curves for MSC cultures established from each of the organs or tissues are shown in Fig. 5. The culture kinetics varied depending on the origin of the cells and the culture stage. In general, the growth rate was low during the early passages, and increased with serial subculture (and time) until a stable value was reached. This behavior was previously reported for bone-marrow-derived murine MSCs alone (da Silva Meirelles and Nardi, 2003). However, differences in the kinetics of the cultures related to the tissue of origin of the MSCs could be observed. The growth rate of brain-derived MSC cultures, for instance, was slow for a longer period when compared with the other cultures. In addition, the stable growth ratios achieved by the different MSC populations, reflected in the inclination of their growth curves, differed.

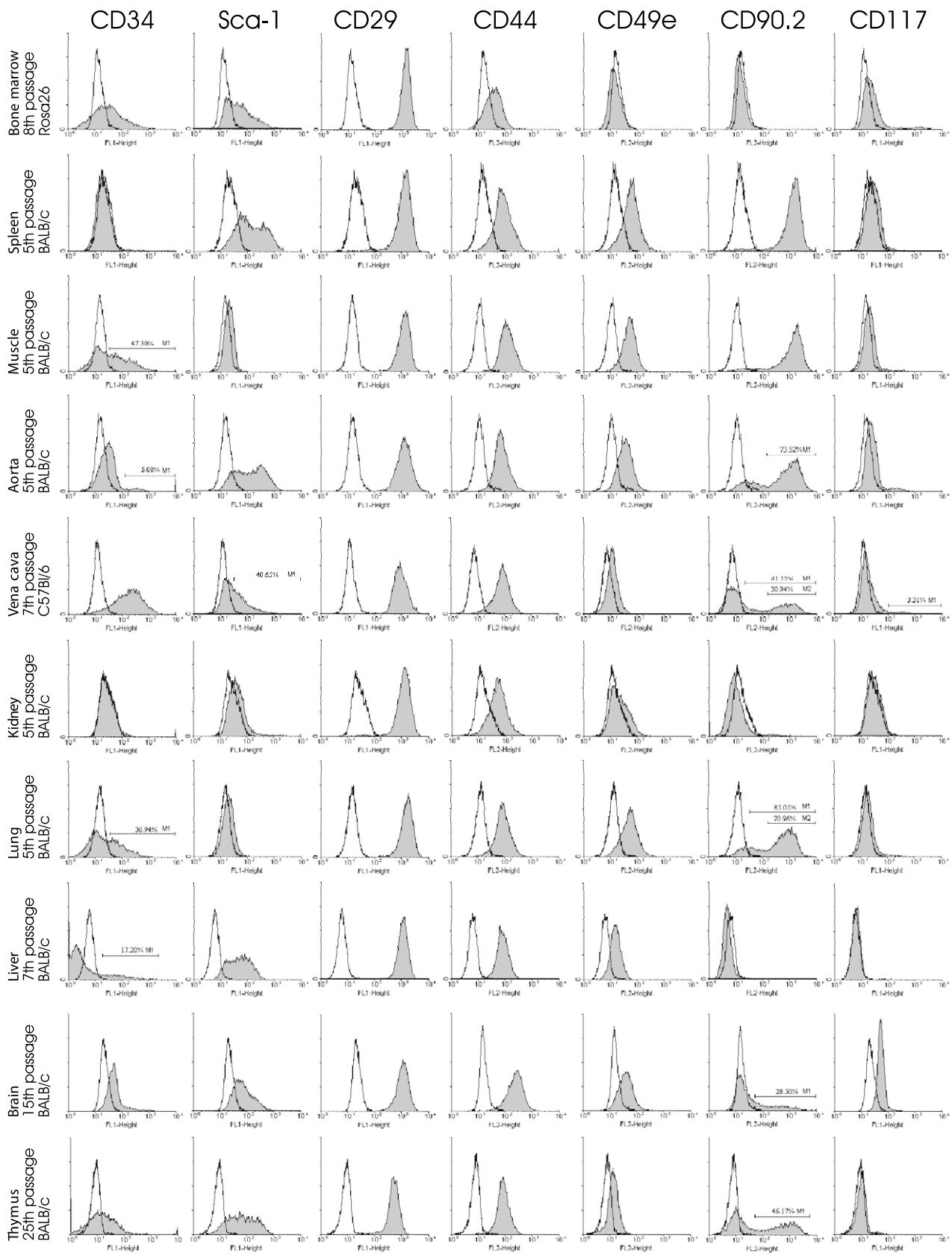

Fig. 2. Immunophenotypic profile of MSCs derived from different sources. Flow cytometry histograms show the expression (shaded) of selected molecules (CD34, Sca-1, CD29, CD44, CD49e, CD90.2 and CD117) by different MSC populations compared with controls (unshaded peaks). Kidney-derived MSCs and kidney glomerulus-derived MSCs share essentially the same surface profile.

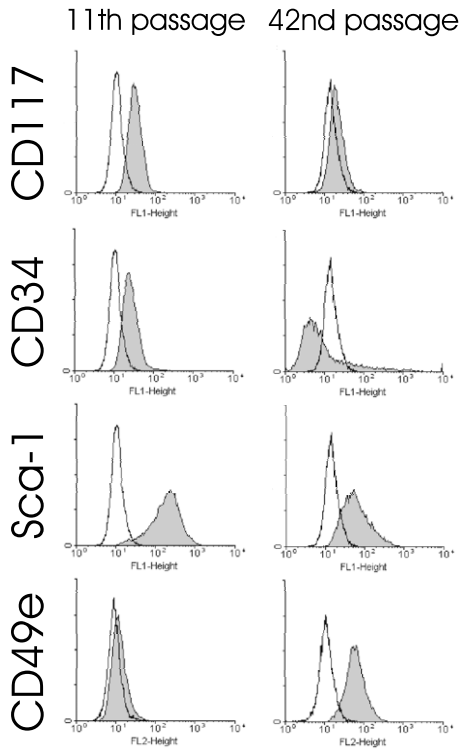

Fig. 3. Effect of long-term culture on the expression of CD117, CD34, Sca-1 and CD49e. Thymus-derived MSCs were analyzed by flow cytometry at passages 11 and 42. Whereas the level of expression of Sca-1 was around 2 log values (versus 1 log control), CD117 expression decreased to nearly control levels, CD34 expression was lost, and expression of CD49e increased 1 log value.

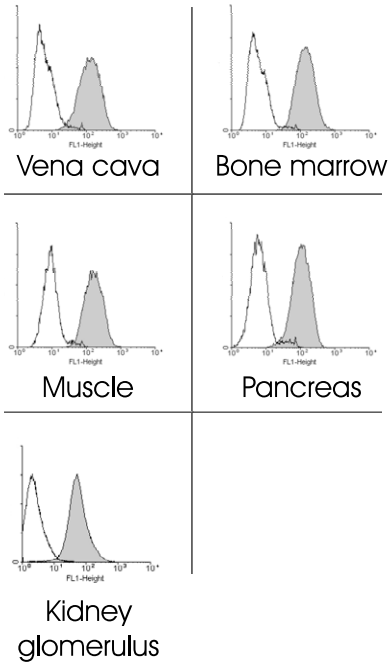

Fig. 4. αSMA expression by MSCs. The histograms represent levels of expression of αSMA (shaded) in MSCs from vena cava, bone marrow, muscle, pancreas and kidney glomeruli compared with controls (unshaded).

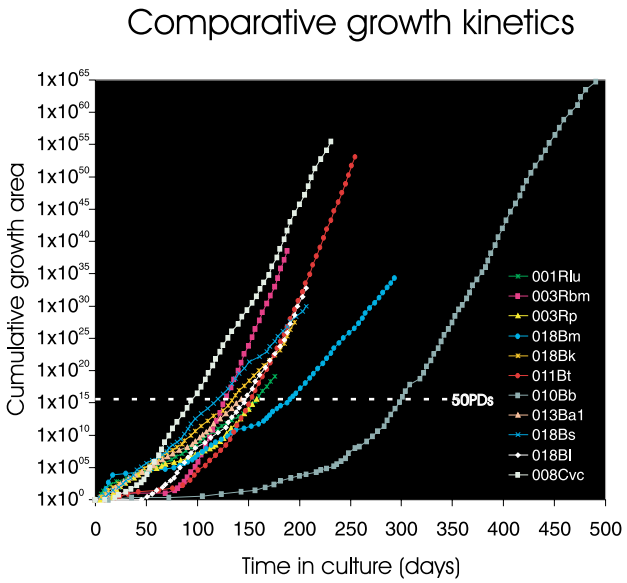

Fig. 5. Comparative growth kinetics of cultures originated from different organs and tissues. Growth curves of representative MSC populations from each source (as presented in Table 1) are plotted. The threshold for 50 population doublings (50 Pds) is shown as a dashed line. Lowercase letters indicate the tissue or organ from which the culture was derived: a, aorta; b, brain; bm, bone marrow; k, kidney; l, liver; lu, lungs; m, muscle; p, pancreas; s, spleen; t, thymus; vc, vena cava. Numbers and capital letters refer to the animal used. Growth area is represented as a multiple of the area occupied by a confluent primary culture, arbitrarily set to 1.

The growth curves shown in Fig. 5 are based on the area occupied by the MSCs. To estimate the expansion in terms of population doublings (PDs), a simple correlation can be made in that 2^{50}, which represents 50 PDs, corresponds to ~1.13×10^{15} times the initial population. On the same basis, 2^{100} equals ~1.27×10^{30} times the initial population, and so on. The MSC populations depicted in Fig. 5 underwent over 50 PDs, some of them reaching 100-200 PDs. We have not observed replicative senescence in any murine MSC long-term cultures. The cultures were generated at different time points and some of them were cultured longer than others. Most of them were cryopreserved by the end of this study.

Differentiation

Functional assays to confirm the MSC identity of the populations studied were preferably performed later (see below), to evaluate the effect of prolonged cultivation on the capacity of the cells to differentiate. When subjected to osteogenic or adipogenic differentiation conditions, the MSC populations confirmed their mesenchymal stem characteristics by depositing a calcium-rich mineralized matrix as evidenced by Alizarin Red S staining, or by acquiring intracellular lipid droplets, evidenced by Oil Red O staining (Fig. 6).

Differences in the frequency of differentiated cells, as well as in the degree of differentiation, could be observed among the cultures originating from different tissues. Vena-cava-derived MSCs, for instance, were very efficient at depositing mineralized matrix, whereas muscle-derived MSCs showed little efficiency (Fig. 6C,G). On the other hand, muscle-derived MSCs were easily induced to differentiate into mature

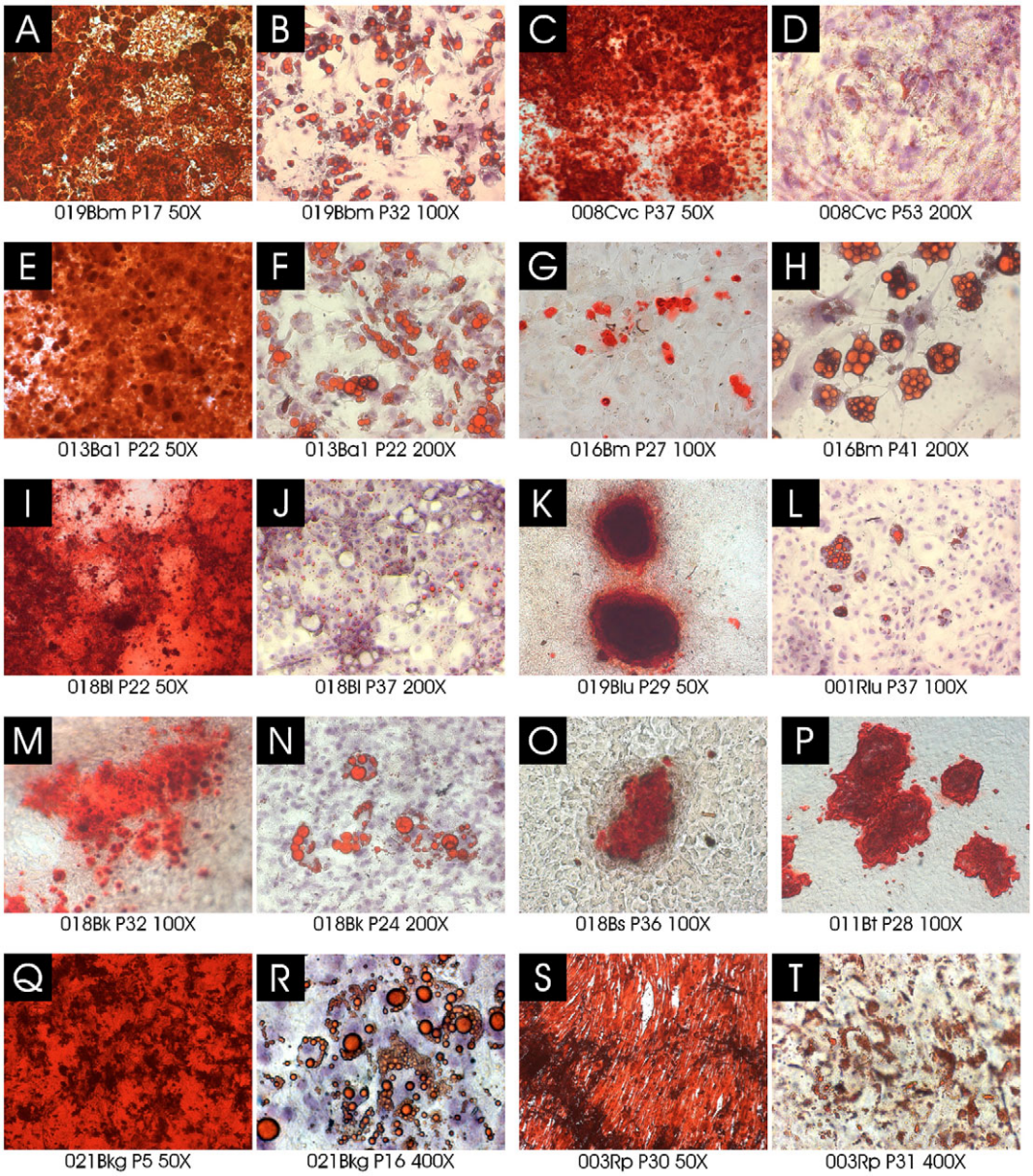

Fig. 6. Differentiation of MSCs derived from different sources, as presented in Table 1. MSCs were cultured in osteogenic or adipogenic medium for up to 2 months. Calcium deposited in the extracellular matrix is stained red by Alizarin Red S (A,C,E,G,I,K,M,O-Q,S). Lipid vacuoles are stained orange with Oil Red O (B,D,F,H,J,L,N,R,T). Magnifications and passage number (P) are indicated below each image. Cell lines are identified as described in the legend to Fig. 5.

adipocytes whereas the vena-cava-derived cultures presented small, poorly developed lipid vacuoles (Fig. 6H,D). Those differences were maintained even when the cultures were exposed to differentiation conditions for longer periods. The differentiation properties of aorta- and bone-marrow-derived MSCs were similar in terms of efficiency and quality (Fig. 6A,B,E,F). Spleen-, thymus-, lung- and kidney-derived MSCs exhibited mineralized nodules when subjected to osteogenic differentiation rather than mineralization of the whole monolayer (Fig. 6K,M,O,P), in contrast to bone-marrow- or aorta-derived MSCs (Fig. 6A,E). The adipogenic differentiation observed in lung-, brain- and kidney-derived MSCs seemed to be less efficient (Fig. 6L,N; brain-derived MSC adipogenic differentiation not shown), even though the degree of adipogenic differentiation presented by kidney- and lung-derived MSCs was comparable to that of bone-marrow-derived MSCs (Fig. 6B). Furthermore, these populations required a longer induction period to differentiate into adipocytes as compared with bone marrow-derived MSCs. Glomeruli-derived MSCs, tested at the fifth passage, differed from the whole-kidney-derived ones regarding osteogenesis: they deposited a rich mineralized matrix that could be detected by the first week of differentiation (Fig. 6Q), and were thus equivalent to bone-marrow-derived MSCs regarding their osteogenic potential. Glomeruli-derived MSCs could differentiate into adipocytes (Fig. 6R), similarly to the whole-kidney-derived cells. Pancreas-derived MSCs also exhibited osteogenic (Fig. 6S) and adipogenic (Fig. 6T) potential.

The protocols used in this study are quite simple, and the use of more complex differentiation strategies (e.g. special substrates and growth factors) might increase the efficiency of differentiation process, so that gross differences may disappear. The results obtained with the assays used in the present study, however, show that the propensity of MSC cultures to respond to different stimuli varies according to their in vivo location.

In addition to differentiation induced as described above, in

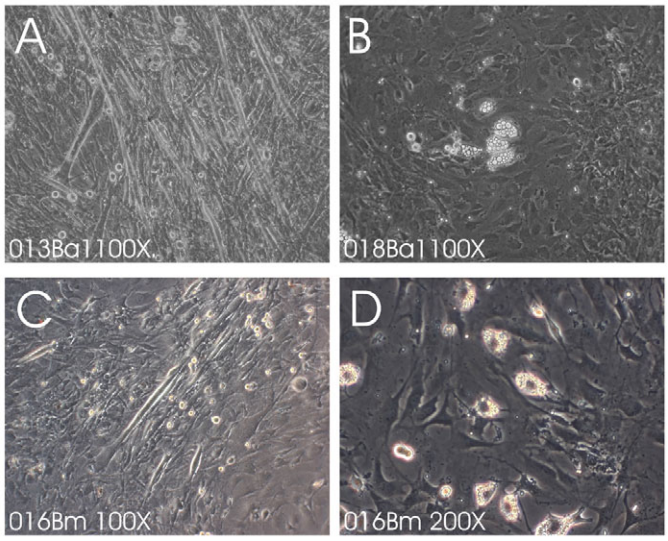

Fig. 7. Non-induced MSC differentiation in primary culture. Aorta primary cultures exhibit myogenic (A) and adipogenic (B) differentiation. The same happens in muscle primary cultures (C and D, respectively). Magnifications are indicated on each image.

some cases spontaneous differentiation was seen in primary cultures. In cultures derived from aorta and muscle, for instance, myotube-like cells and adipocytes were often observed (Fig. 7). This phenomenon could be caused by the short-term exposure to amphotericin-B present in antibiotic-antimycotic solution (Phinney et al., 1999), or by the presence of myogenic- and adipogenic-committed progenitors in the primary culture. Spontaneous differentiation was not observed in primary cultures originating from the other organs and tissues studied.

Cloning

Two cloning processes were performed, with long-term MSC cultures. In each of them, three 96-well plates were seeded with individual cells and analyzed 2 weeks later. The results were very similar, as presented in Table 2, and showed that around 50% of the cells were able to originate clones, with different potentials for expansion. The morphology of the clones was the same observed for the MSC cultures. Eight of the clones were selected for further expansion, and were able to establish cultures with normal growth kinetics, maintained for around 4 months. Two of these cultures were subcloned, and one of the subclones was submitted to a further subcloning process, with results similar to those of the initial cloning (Table 2). One clone from each cloning procedure was tested for the ability to differentiate along osteogenic or adipogenic pathways and exhibited differentiation capabilities similar to those of parental cultures in terms of both efficiency and quality.

Discussion

Mesenchymal stem cells have been conventionally isolated from bone marrow (Pittenger et al., 1999; Kopen et al., 1999) and, more recently, from some other tissues (Zuk et al., 2001; De Bari et al., 2001; Seo et al., 2004; Sabatini et al., 2005). This study was originally designed to investigate whether, using the same conditions established for the cultivation of murine bone-marrow-derived MSCs (da Silva Meirelles and Nardi, 2003), these cells could be found in other organs. Surprisingly, the results showed that long-term MSC cultures could be established from all the organs and tissues studied, irrespective or their embryonic origin. The cell populations thus obtained can be operationally defined as MSCs, because they exhibit the capacity of prolonged self-renewal and differentiate along mesenchymal cell lineages. The possibility that the long-term cultures might represent early progenitors exhibiting multipotent capabilities was tested with cloning experiments. The results showed that a percentage of cells plated at the single-cell level were able to regenerate long-term cell cultures indicating that, at a given time point, a proportion of the cells in each population is committed to self-renewal whereas the remainder are not. When clones were cultured under osteogenic or adipogenic conditions, they exhibited differentiation characteristics similar to those of parental cultures. This indicates that the long-term cultures represent populations containing stem cells, and suggests that population asymmetry is responsible for the maintenance of the stem cell pool (Watt and Hogan, 2000).

The MSC cell populations originating from brain, spleen, liver, kidney, kidney glomeruli, lung, bone marrow, muscle, thymus and pancreas presented similar morphology and, to a certain extent, surface marker profile. On the other hand, the differentiation assays showed some variation among the cultures in the frequency of cells which actually differentiated

Table 2. Results of cloning and subcloning of long-term MSC cultures

	Culture				
	Cloning 1	Cloning 2	Subcloning 1	Subcloning 2	Sub-subcloning
–	112 (38.8)	144 (50.0)	61 (31.8)	42 (21.9)	25 (26.0)
+	36 (12.5)	12 (4.2)	34 (17.7)	53 (27.6)	16 (16.7)
++	75 (26.0)	84 (29.2)	35 (18.2)	21 (10.9)	n.a.
+++	49 (17.0)	39 (13.5)	51 (26.6)	73 (38.0)	55 (57.3)
Aborted	12 (4.2)	6 (2.1)	9 (4.7)	3 (1.6)	–
Differentiated	4 (1.5)	3 (1.0)	2 (1.0)	–	–
Osteogenic differentiation	+ (Clone H4)	+ (Clone 3D10)	n.a.	n.a.	n.a.
Adipogenic differentiation	+ (Clone H4)	+ (Clone 3D10)	n.a.	n.a.	n.a.

MSC long-term cultures were cloned by micromanipulation. Individual cells were plated in 96-well plates (three plates for each cloning process) with MSC-conditioned medium and maintained for 2 weeks. Two well-developed clones were subcloned (two plates for each process), and one of the resulting cultures was further subcloned (one plate prepared). The plates were analyzed for the number (and percentage) of negative (–) and positive wells which were classified according to the size of the colonies (+, ++, +++). Aborted clones were those in which the cells proliferated and then spontaneously died. Differentiated clones developed mature morphologies, which were not further investigated. n.a., not applicable.

in the osteogenic or adipogenic phenotype, as well as on the degree of differentiation, related to their site of origin. This might be due to the influence of the local environment from which they originate, reflecting the importance of the niche in establishing the phenotype of the stem cells it interacts with (Fuchs et al., 2004).

Whereas most other studies have approached the question of the natural distribution of MSCs in the organism by infusing cultured cells into the animal models and analyzing their multi-site engraftment, this is, to our knowledge, the first one to apply the opposite approach. The simultaneous analysis of different organs and tissues for their MSC contents can provide more accurate information regarding their natural in vivo distribution. The wide distribution of MSCs observed raised the question of the relationship among these populations. Three hypotheses were then considered: first, MSCs are tissue-resident cells, and can be collected from individual tissues or organs; second, MSCs are resident in tissues, and circulate in blood; third, MSCs are derived from the circulating blood.

To test these hypotheses, the possibility that the MSC long-term cultures were derived from cells circulating in the blood was first analyzed by perfusing the animals before collection of the tissues or organs. Cultures could be normally established under these conditions. Since no long-term culture could be derived from circulating blood collected under controlled conditions, MSCs seem to be absent from the circulation under normal physiological conditions, in agreement with Wexler et al. (Wexler et al., 2003). The possibility that they circulate systemically or locally under other circumstances, e.g. during tissue injury, is not however excluded and was not tested in the present study. Endothelial progenitor cells, for instance, can be found at higher frequency in the peripheral blood of patients with acute myocardial infarction (Shintani et al., 2001).

The characteristics of MSC populations obtained from the different organs were however very similar, suggesting a closer relationship between them. Since literature reports have suggested that MSCs derive from perivascular cells (Doherty et al., 1998; Bianco et al., 2001; Shi and Gronthos, 2003; Farrington-Rock et al., 2004), it is possible that MSCs are actually derived from the vasculature. To test this hypothesis, the aorta and the vena cava were investigated and long-term MSC cultures could be established from both tissues. We next analyzed these and the MSC populations obtained from other sources and found them positive for the vascular smooth muscle cell marker αSMA (Owens, 1995). To demonstrate that perivascular cells at the capillary level have mesenchymal stem cell properties, we isolated a structure comprising capillaries only – the kidney glomerulus. Decapsulated glomeruli are composed of endothelial cells, podocytes and mesangial cells – which are considered specialized pericytes (Schlondorff, 1987). Based on the expression of αSMA, reported as a marker for activated mesangial cells (Johnson et al., 1991), lack of expression of CD31 by the resultant population, and morphology, the cultures can be regarded as originating from mesangial cells within the glomerulus. Their osteogenic and adipogenic differentiation capabilities, along with their self-renewal capacity, allow them to be operationally defined as MSCs. Taking these results as a whole, we conclude that the MSC compartment extends through the whole post-natal organism as a result of its perivascular location.

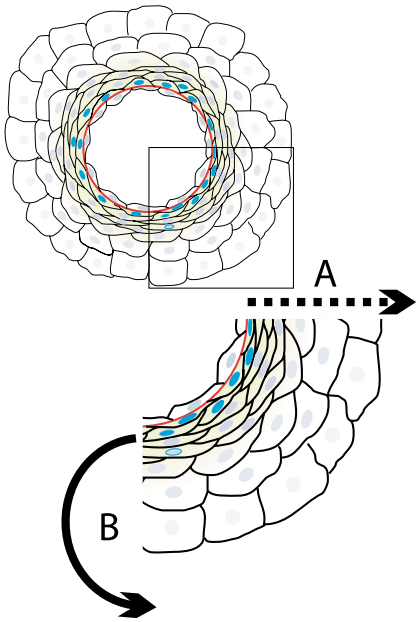

Fig. 8. A proposed model of MSC contribution to tissue maintenance. In this schematic representation of the transverse section of a simple vessel, MSCs lie in the basement membrane (red line), opposed to endothelial cells. Cues provided by the tissue-specific microenvironment coordinate a gradual transition (represented by green color gradient) from undifferentiated cells to progenitor and mature cell phenotypes. This process can occur naturally as represented by the dotted arrow (A). In case of tissue injury, undifferentiated MSCs can be mobilized directly into the tissue without the progenitor transition as represented by the curved arrow (B).

Early in vivo experiments have suggested that pericytes may act as a source of undifferentiated cells during adipose (Richardson et al., 1982) and osseous (Diaz-Flores et al., 1992) tissue repair. These data, along with reports describing the MSC differentiation capabilities (Pittenger et al., 1999; Kopen et al., 1999; Woodbury et al., 2000; Sato et al., 2005; Choi et al., 2005; Lange et al., 2005), and the functional differences between the populations studied here according to their origin, led us to propose the model depicted in Fig. 8. In this model, MSCs act as a reservoir of undifferentiated cells to supply the cellular (and non-cellular) demands of the tissue they belong to, acquiring local phenotypic characteristics. When necessary, and after signs from the microenvironment, they give rise to committed progenitors that gradually integrate into the tissue (Fig. 8A). Tissue injury can activate alternative processes (Fig. 8B). The model does not exclude the possible existence of other tissue-specific stem cells; however, it suggests that a portion of the apparent post-natal stem cell diversity may be attributed to local MSCs behaving as tissue-specific stem cells. Once again the term ‘mesenchymal’ stem cell seems inappropriate, and possibly the term ‘perivascular stem cell’ might best represent this particular cell type.

We believe that, in addition to providing insight into MSC biology, our findings and hypotheses can be useful for designing therapeutic strategies for a range of diseases. Irradiation, or drugs able to transiently destabilize the vessel wall integrity, might facilitate cell engraftment in cell or cell-

mediated therapies; also, drugs and genetic therapy vectors could be directed to the perivascular compartment to achieve tissue-specific activity, as perivascular-derived cells gradually assume a tissue-specific phenotype. These approaches validate the circulatory system and more specifically its stem cell compartment as a vehicle for reaching the whole organism.

Materials and Methods

Reagents, culture media and solutions

Complete culture medium (CCM) was composed of Dulbecco's modified Eagle's medium (DMEM) with HEPES (free acid, 2.5–3.7 g/l) and 10% fetal bovine serum (Cultilab, Sao Paulo, Brazil). Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution containing 10 mM sodium HEPES (HB-CMF-HBSS) combined with DMEM (1:1) was used as perfusion medium. All reagents used in this study were from Sigma Chemical Co. (St Louis, MO), unless otherwise stated. Plasticware was from TPP (Trasadingen, Switzerland).

Animals

Adult mice (8-43 weeks old) from the C57Bl/6 and BALB/c strains were used in this study. ROSA26 (The Jackson Laboratory, Bar Harbor, ME) and eGFP mice (green mouse FM131, kindly provided by M. Okabe, Osaka University, Japan), derived from the C57Bl/6 strain, were also used. The animals were kept under standard conditions (12 hours light/12 hours dark, water and food ad libitum) in our animal house. All the experimental procedures were performed according to institutional guidelines.

Perfusion

The animals were anesthetized with a combination of ketamine and xilazine (1.16 g and 2.3 g per kg body weight, respectively) delivered intraperitoneally. The abdominal cavity was opened, the diaphragm was ruptured, and 100 units of heparin in 200 μl HB-CMF-HBSS were injected into the beating heart. The ascending aorta was catheterized with a 27G intravenous catheter inserted through the left ventricle. The caudal vena cava was cut, and around 50 ml of perfusion medium were pumped in. For lung perfusion, the pulmonary artery was catheterized instead.

MSC isolation and long-term culture

MSCs from bone marrow were isolated and cultured as previously described (da Silva Meirelles and Nardi, 2003). MSCs from liver, spleen, pancreas, lung, kidney, aorta, vena cava, brain and muscle were obtained as follows. Organs and tissues were collected from perfused or non-perfused animals, rinsed in HB-CMF-HBSS, transferred to a Petri dish and cut into small pieces. When dissecting organs, care was taken to discard the portions containing visible vessels (e.g. the portal vein and the vena cava in the liver). The dissected pieces (around 0.2-0.8 cm^3) were washed with HB-CMF-HBSS, cut into smaller fragments, and subsequently digested with collagenase type I (0.5 mg/ml in DMEM/10 mM HEPES) for 30 minutes to 3 hours at 37°C. To separate the adipose layer surrounding the aorta, the vessel was digested for around 30 minutes and subjected to vigorous agitation, yielding a first cell fraction. The remnant of the vessel was then washed in 20 ml HB-CMF-HBSS, and transferred to a new tube where digestion proceeded yielding a second cell fraction. Both cell fractions were used to establish separate primary cultures.

Whenever gross remnants persisted after collagenase digestion, they were allowed to settle for 1 to 3 minutes, and the supernatant was transferred to a new tube which was then completed with CCM. In some experiments, the cells were further cleared from debris by centrifugation on Ficoll-Hypaque (Amersham Pharmacia, Piscataway, NJ), followed by an additional washing step. After centrifugation at 400 ***g*** for 10 minutes at room temperature (RT), the pellets were resuspended in 3.5 ml CCM containing 1% antibiotic-antimycotic solution (GIBCO BRL, Gaithersburg, MD), seeded in six-well dishes (3.5 ml/well) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Three days later, if the cultures were not confluent, the whole volume of CCM (with no antibiotics or antimycotics) was replaced, and the adherent layer was refed every 3 or 4 days.

For subculture, the adherent layer was washed once and incubated with 0.25% trypsin and 0.01% EDTA in HB-CMF-HBSS. The cultures were split whenever they reached confluence, at ratios empirically determined for two subcultures a week at most. Initial split ratios were 1:2 or 1:3, and as the culture kinetics accelerated the ratios were set to values ranging from 1:6 to 1:25, until they stabilized at different ratios as described below.

To evaluate the presence of MSCs in blood, animals were anesthetized, the abdominal cavity was opened, and 100 units of heparin in 200 μl HB-CMF-HBSS were injected into the beating heart. Either a 27G intravenous catheter was introduced cranially into the portal vein and 500-750 μl blood were collected, or the vessels arising from the heart were cut and 500-750 μl blood were collected from the thoracic cavity. Blood was also collected directly from the exposed heart in some cases. The collected blood was either added to a 25 cm^2 flask containing 7 ml CCM incubated at 37°C, or fractionated on Ficoll-Hypaque. In this case, mononuclear cells were collected, washed once in complete medium, resuspended in 3.5 ml fresh complete medium, transferred to a well of a six-well dish and incubated at 37°C. In either case, after 3 days, non-adherent cells were removed along with the culture medium and fresh complete medium was added. The adherent cells were then re-fed every 3 or 4 days.

To establish glomeruli-derived MSC cultures, kidneys were placed into a 15 ml centrifuge tube containing 5 ml CCM, and mechanically disrupted by several rounds of aspiration/expulsion using a 10 ml pipette. Single glomeruli devoid of the Bowman's capsule were isolated from the cell suspension by micromanipulation, and transferred either individually or collectively to 12-well dishes containing CCM and 1% antibiotic-antimycotic solution. Subsequent passages were performed as described above.

Cell cloning

To clone MSCs to the single-cell level, cultures were trypsinized, resuspended in MSC-conditioned medium which had been previously filtered through a 0.22 μm membrane, and individually transferred to 96-well dishes using a micromanipulator. The number, morphology and kinetics of resulting clones were analyzed, and some of them were selected for subcloning and differentiation assays.

Morphological analysis and photographs

MSC cultures were routinely observed with an inverted phase-contrast microscope (Axiovert 25; Zeiss, Hallbergmoos, Germany). For detailed observation, cells were rinsed with phosphate-buffered saline (PBS), fixed with ethanol for 5 minutes at RT in some cases, and stained for 2.5 minutes with Giemsa. Photomicrographs were taken with a digital camera (AxioCam MRc, Zeiss), using AxioVision 3.1 software (Zeiss).

Flow cytometry

For detection of surface antigens the cells were trypsinized, centrifuged, and incubated for 30 minutes at 4°C with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies against murine Sca-1, Gr-1, CD11b, CD13, CD18, CD19, CD29, CD31, CD44, CD45, CD49d, CD49e, CD90.2, CD117 and IgG (Pharmingen BD, San Diego, CA). Excess antibody was removed by washing.

For the detection of α-smooth muscle actin (αSMA), the cells were collected, washed once in HB-CMF-HBSS and fixed with 4% paraformaldehyde in PBS for 1 hour at RT. After centrifugation, the cells were kept for 15 minutes at RT in 5 ml PBS containing 0.2% Triton X-100. Cells were collected, washed once in PBS, and incubated with or without primary antibody against αSMA (Chemicon, Temecula, CA) overnight at 4°C. The cells were then incubated with FITC-conjugated anti-mouse IgG secondary antibody for 1 hour at 4°C.

The cells were analyzed using a FACScalibur cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA) with the CellQuest software. At least 10,000 events were collected. The WinMDI 2.8 software was used for building histograms.

MSC differentiation

Osteogenic differentiation was induced by culturing MSCs for up to 8 weeks in CCM supplemented with 10^{-8} M dexamethasone, 5 μg/ml ascorbic acid 2-phosphate and 10 mM β-glycerophosphate (Phinney et al., 1999). To observe calcium deposition, cultures were washed once with PBS, fixed with 4% paraformaldehyde in PBS for 15-30 minutes at RT, and stained for 5 minutes at RT with Alizarin Red S stain at pH 4.2. Excess stain was removed by several washes with distilled water.

To induce adipogenic differentiation, MSCs were cultured for up to 8 weeks in CCM supplemented with 10^{-8} M dexamethasone, 2.5 μg/ml insulin, 100 μM indomethacin and, in some experiments, 3.5 μM rosiglitazone or 5 μM 15-deoxy-$D^{12,14}$-prostaglandin J_2. Later in this study, DMEM with 10 mM HEPES, heparin and 20% platelet-free human plasma (Krawisz and Scott, 1982) was used to induce kidney glomerulus-derived MSC adipogenic differentiation. Adipocytes were easily discerned from the undifferentiated cells by phase-contrast microscopy. To further confirm their identity, cells were fixed with 4% paraformaldehyde in PBS for 1 hour at RT, and stained with either Oil Red O solution (three volumes of 3.75% Oil Red O in isopropanol plus two volumes of distilled water) or Sudan Black B solution (three volumes of 2% Sudan Black B in isopropanol plus two volumes of distilled water) for 5 minutes at RT. When stained with Oil Red O, the cultures were counterstained with Harry's hematoxylin (1 minute at RT).

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