Nonhematopoietic/endothelial SSEA-1+ cells define the most primitive progenitors in the adult murine bone marrow mesenchymal compartment

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Introduction

The adult bone marrow has generally been seen as an organ composed of 2 main and distinct lineages: the hematopoietic tissue and a group of mesenchymal-derived cells. It is believed that the bone marrow mesenchymal compartment contains stem/progenitors of skeletal tissue components such as bone, cartilage, hematopoietic-supporting stroma, and adipocytes. These cells are currently named as mesenchymal stem or progenitor cells (MSCs/MPCs).

Since the first description of MSCs in the early 1970s, researchers have failed to isolate the most primitive cell type from bulk cultures of mesenchymal cells obtained by adherence to plastic. Through in vitro studies and accumulative observations throughout the years, the notion is that ex vivo–expanded mesenchymal stromal cells (MStrCs) are indeed heterogeneous and composed mainly of mesenchymal cells (without any stem/progenitor properties) and potentially rare “true” stem/progenitor cells. Several groups have reported the use of different cell-surface antigens to isolate/enrich different mesenchymal progenitors with restricted differentiation potentials. However, there is no defined universal stem/progenitor cell marker yet that could be used to isolate/enrich the putative most primitive mesenchymal subset with high differentiation capacities both in vitro and in vivo. The only bone marrow–derived cell type known to date with such capacities is the multipotential adult progenitor cells (MAPCs). However, these multipotent cells were not prospectively isolated, thus their existence as such in vivo is still uncertain.

Herein, we are reporting the identification, isolation, and detailed characterization of a subpopulation of cells in the murine mesenchymal compartment (Lin/CD45/CD31- and CD45/CD11b- fractions from bone marrow and adherent cultures, respectively) based on the expression of SSEA-1. Using several experimental approaches we show that SSEA-1+ cells are primitive cells, as opposed to the SSEA-1- mesenchymal population, and thus we are proposing that SSEA-1+ cells are the most primitive mesenchymal subset described so far.

Materials and methods

Isolation, purification, and expansion of bulk and different subpopulations of mesenchymal cells (MCs)

Bone marrow cells were collected from 6- to 10-week-old NOD/LtSz-scid/Scid mice (nonobese diabetic–severe combined immunodeficiency [NOD/SCID]). Isolation and enrichment procedures of mesenchymal cell cultures were performed as previously described.

To identify SSEA-1+ MCs in the mouse bone marrow, lineage-negative cells were obtained (from 2- to 14-week-old mice) using the murine lineage cocktail from Stem Cell Technologies (Vancouver, BC, Canada) according to the manufacturer’s instructions. Lineage (Lin)–negative cells were further stained with anti-CD31, anti-CD45 (both from Pharmingen, Oxford, United Kingdom), and anti–SSEA-1 (Developmental Studies Hybridoma Bank, Iowa City, IA), and cells were analyzed based on Lin/CD45/CD31- and SSEA-1+ expression using a Life Science Research (LSR; Becton Dickinson, Oxford, United Kingdom). DAPI was used for exclusion of dead cells.

Isolation of SSEA-1+ cells was performed using fluorescence-activated cell sorter (FACS) sorting after the cells had been labeled with SSEA-1 antibody followed by anti–mouse IgM-PE antibody (Southern Biotechnologies, Birmingham, AL). For SSEA-1+ MC enrichment we used anti-PE immunomagnetic beads according to the manufacturer’s instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). For clonal analysis, single SSEA-1+ cells were sorted from passage 1–adherent cultures into single
wells of 96-well plates. Culturing SSEA-1+ MCs in MAPC condition was performed as described by others.9,10 Briefly, SSEA-1+ MCs were cultured on 1 μg/cm² of fibronectin (Sigma, Poole, United Kingdom) and cells were maintained at a density of 500 to 2500 cells/cm².

In vitro differentiation

Cells were plated at a density of 5000 cells/cm² with their respective expansion medium for 24 hours. After attachment, cells were washed once with basic differentiation medium consisting of DMEM 2% FBS and antibiotics and then replaced with the same medium containing specific differentiation supplements.9,10,12-14 The specific supplements for each lineage were osteogenic (50 μM ascorbic acid 2-phosphate, 100 mM Dex, and 10 mM β-glycerophosphate); adipogenic (1 μM Dexamethasone, 50 μM indomethacin, 500 mM 3-isobutyl-1-methylxanthine, and 5 μg/mL of insulin or 5% horse serum [HS]); myogenic (10 μM 5'-azacytidine, 50 μM hydrocortisone and 5% HS for the initial 24-hour period, and then maintained in the same conditions without 5'-azacytidine: endoderm (hepatocyte-like cells; 10 ng/mL of HGF and FGF-4); and neuroectoderm (astrocyte-like cells; 50 ng/mL of bFGF and 20 ng/mL of mEFG). All chemicals were from Sigma and cytokines were from Peprotech (London, United Kingdom).

Immunocytochemistry

To evaluate osteogenic potential, alkaline-phosphatase activity was determined as recommended by the manufacturer’s instructions contained in Sigma kit number 85. For adipogenic determination, Oil Red O staining was used. Briefly, for immunocytochemistry, cells were fixed with 4% paraformaldehyde at room temperature, and sequentially stained with primary antibodies at 10 minutes, blocked with 10% serum (rat and/or goat) for 30 minutes at room temperature, and sequentially stained with primary antibodies at 4°C overnight (ON), and then with appropriate secondary antibodies for 1 hour at room temperature. Between steps, slides were washed twice with PBS 2% BSA. Intracellular staining for FACS analysis was performed in a similar fashion, with primary/secondary antibody steps performed at room temperature for 30 minutes or 60 minutes each for cytoplasmic or nuclear antigens, respectively. Between steps, cells were washed twice with PBS 2% FBS.

Transplantation in animals

All animal procedures were performed in accordance with Cancer Research UK (CRUK) institutional and UK Home Office guidelines. Transplantation using newborn mice was performed as follows: unmanipulated clone-3 SSEA-1+ cells (2 × 10⁵ cells at 28 doublings) were injected intravenously (facial vein) into 72-day-old NOD/SCID–/– mice; animals were maintained for 5 to 8 weeks and then killed. Transplantation using adult mice was performed as follows: clone-3 cells (at 32 doublings) were first lentivirally transduced (with MOI 30) as described previously;11 prior to injection, mice (NOD/SCID) were first anesthetized; purified GFP- SSEA-1+ cells (3 × 10⁶ cells at 38 doublings) were inoculated into the right tibia of each sublethally irradiated mouse (375 cGy; 137Cs source); animals were maintained for 6 weeks and killed.

Tissue processing and immunohistochemistry

Bone marrow cells were collected by flushing the femora, tibiae, and iliac crests. Bone marrow mononuclear cell (BMMNC) suspensions were obtained after all cells had been subjected to red-cell lysis. Cells were then stained with specific cell-surface antibodies and analyzed by FACS.

Tibiae and other bones were fixed in 10% neutral buffered formaldehyde ON at 4°C. On the following day, fixed bones were decalcified in 100 mM EDTA (Sigma, Poole, United Kingdom) and sequentially stained with primary antibodies at 4°C overnight (ON), and then with appropriate secondary antibodies for 1 hour at room temperature. Between steps, slides were washed twice with PBS 2% BSA. Intracellular staining for FACS analysis was performed in a similar fashion, with primary/secondary antibody steps performed at room temperature for 30 minutes or 60 minutes each for cytoplasmic or nuclear antigens, respectively. Between steps, cells were washed twice with PBS 2% FBS.

Reverse transcriptase–polymerase chain reaction (RT-PCR) and quantitative RT-PCR (QRT-PCR)

RNA was extracted using the RNAeasy Micro Kit and reverse transcribed using the Senscript or Omniscript Kits according to the manufacturer’s instructions (Qiagen, Crawley, United Kingdom). Also, DNase treatment was performed to cleanup possible genomic DNA contamination. For RT-PCR, amplification (5 to 25 ng cDNA/25 μL reaction) was performed using the Taq PCR Core Kit (Qiagen) with the following conditions: 95°C for 30 minutes, 60°C for 1 hour, for 40 cycles. Correct size of PCR products was confirmed by separation on a 2% agarose gel. For quantitative real-time PCR (5 to 25 ng cDNA/25 μL reaction), SybrGreen master mix reagent (Applied Biosystems, Warrington, United Kingdom) was used according to the manufacturer’s instruction. Amplification was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems). To avoid the possibility of amplifying contaminating DNA and unprocessed mRNA, most primers were designed (when possible) to anneal the end parts of 2 distant exons, and therefore each amplicon covers at least 2 or more exons without any introns. The specificity of the PCR products was verified by running a 2% agarose gel and also using the dissociation curve V1.0 software (Applied Biosystems).

Karyotyping

For karyotyping, cultures were exposed to 0.1 μg/mL colcemid (Sigma) for 12 hours, harvested, washed, and exposed to 75-mM KCl hypotonic solution for 15 minutes at 37°C. Hypotonic solution was washed out by centrifugation and cells were fixed with ice-cold methanol/acetic acid (3:1) fixative. Fixed cells were collected by centrifugation and resuspended in the same fixative solution. Cells were dropped onto a cold glass slide. Chromosomes were stained using a solution containing DAPI. Thirty and 50 metaphases were analyzed for clone-3 and adherent passage 1 mesenchymal cell populations, respectively. Modal distribution analysis was performed to qualitify the karyotype.

Cell-cycle analysis with Hst/PY

Different cell types were resuspended at a density of 10⁶ cells/mL of Hst (Hst) buffer containing 5 mMol/mL of Hst (Probes). Hst buffer consisted of Hanks balanced salt solution (HANKS), 20 μM of HEPES (Gibco, Paisley, United Kingdom), 1 mM of glucose, and 10% FBS. After incubation at 37°C for 45 minutes, Pyronin-Y (PY; Sigma) was added to a final concentration of 2.5 μg/mL for an additional 45 minutes at 37°C. Cells were washed once and resuspended in Hst buffer and then analyzed. In some cases, antibody staining was performed after the Hst/PY step and was carried out in Hst buffer containing the 2 dyes. Pulse processing was used in order to exclude any unstained, apoptotic, and clumped cells and analysis was performed using an LSR (Becton Dickinson).
Results

Identification of SSEA-1+ cells in the bone marrow mesenchymal compartment both in vitro and in vivo

In order to minimize possible culture artifacts that could alter any cell fate, we used a simple method to enrich and expand MStrCs based on their ability to adhere to plastic followed by negative selection using CD45 and CD11b antigens to exclude all the hematopoietic contaminants from early cultures. After enrichment, we first verified their in vitro differentiation capacities. Upon appropriate culture conditions, some mesenchymal cells were able to give rise not only to adipocytes, osteoblasts, and myoblasts but also to unconventional cells like astrocyte/neuronal-, endothelial-, and hepatocyte-like cells. This was determined by lineage-specific immunocytochemistry stainings (Figure S1) and RT-PCR studies (data not shown).

During the course of our experiments we observed that only a fraction of the cells were capable of differentiation to a specific pathway. Our in vitro results are in accordance with the notion that MStrCs are heterogeneous. Nonetheless, we could not identify any subpopulations based on several markers reported to be expressed on MStrCs from different species (eg, Sca-1, CD44, CD105, CD73, CD106). This small and quiescent subpopulation displays a phenotype (eg, expressing KDR/VEGF-R2 and Trk/NGF-R) that is different from the most abundant, fast-growing, and committed precursors. We observed that Trk- and KDR-expressing cells comprised between 0.5% and 1.2% and 0.4% and 0.8% of bulk mesenchymal cultures (from 4 different extractions), respectively. Interestingly, all KDR+ cells coexisted with Trk (Figure 1A) and these cells were small and agranular (data not shown). These data indicate that bulk murine mesenchymal cultures do effectively contain RS1 cells.

Next, we went on to investigate whether these cultures also contain any SSEA-1+ cells as described for MAPCs. We found an SSEA-1+ subpopulation, comprising 0.45% to 0.97% of all mesenchymal cells (n = 6; Figure 1B). Further analysis of the cells also revealed that the majority of the quiescent (G0) cells (in confluent cultures) were composed mainly of SSEA-1+ cells (Figure 1C). Moreover, we observed that the SSEA-1+ population is still heterogeneous and includes not only all the RS1 cells (KDR/Trk+) but also other cell types. The SSEA-1/KDR/Trk+ fraction comprised around 70% to 80% of the SSEA-1+ fraction, whereas the second major fraction that was negative for KDR and Trk (Figure 1B) comprised less than 25% (n = 4) of this population. A third small fraction could occasionally be identified expressing only Trk.

Although we were able to isolate and identify a subpopulation of cells in mesenchymal cell cultures with expression of an embryonic antigen, the question of their true existence directly in the bone marrow remained. We were able to identify an SSEA-1+ population in the putative mesenchymal compartment (Figure 1D, R1) that comprised approximately 0.04% of the Lin/CD45/CD31− fraction in an adult mouse (aged >12 weeks). Interestingly, we saw that this nonhematopoietic/endothelial SSEA-1+ population decreased with age (Figure 1E), showing a variation during mouse ontogeny. This population also expressed low levels of Sca-1 (Figure 1D). In addition, this population could be located in situ in the bone marrow in close vicinity to CD45/CD11b+/TER119/ CD31+ cells and slightly enriched below the endochondral-bone region in a developing bone (Figure S2).

We further asked whether this newly identified bone marrow–derived subpopulation expressed other key transcription factors for the maintenance of the undifferentiated state of ES. We were able to isolate and identify a subpopulation of cells in mesenchymal cell cultures with expression of an embryonic antigen, the question of their true existence directly in the bone marrow remained. We were able to identify an SSEA-1+ population in the putative mesenchymal compartment (Figure 1D, R1) that comprised approximately 0.04% of the Lin/CD45/CD31− fraction in an adult mouse (aged >12 weeks). Interestingly, we saw that this nonhematopoietic/endothelial SSEA-1+ population decreased with age (Figure 1E), showing a variation during mouse ontogeny. This population also expressed low levels of Sca-1 (Figure 1D). In addition, this population could be located in situ in the bone marrow in close vicinity to CD45/CD11b+/TER119/ CD31+ cells and slightly enriched below the endochondral-bone region in a developing bone (Figure S2).

Figure 1. Revealing the hierarchical organization of the murine mesenchymal compartment. (A-C) Representative flow-cytometry plots of mesenchymal cell (MC) cultures from passage 5 (P5). (A) Bulk mesenchymal cultures contain RS1-like cells. (B) SSEA-1 is expressed by some mesenchymal cells and this population is still heterogeneous for KDR and Trk expressions. (C) Hst/PY staining showing that the quiescent (G0) cell subpopulation is highly enriched for SSEA-1–expressing cells. (D) Identification of SSEA-1–expressing cells directly in bone marrow cells. Lineage-depleted BMMNCs were stained for Lin/CD45/CD31, SSEA-1, and Sca-1. Representative FACS-staining profile of cells derived from 2-week-old mice. The negative fraction for the expression of Lin/CD45/CD31 was gated (R1) and plotted for SSEA-1 and Sca-1 expressions. (E) Frequency variation of SSEA-1+ cells during mouse ontogeny. The frequency was calculated by multiplying the percentage of mesenchymal cells in BMMNCs (R1) with that of SSEA-1+ cells in the mesenchymal population (R2) obtained for each group of mice. The analysis was performed on pooled samples of BMMNCs from 8 (2 weeks old), 10 (4 weeks old), 6 (6 weeks old), and 3 (>12 weeks old; n = 2) mice, respectively, for each group. (F) Expression of Oct-3/4 and Nanog determined by QRT-PCR in SSEA-1− (S−) and SSEA-1+ (S+) cells isolated from MC cultures and directly from the bone marrow (from R1 fraction); representative gel electrophoresis with GAPDH as the internal PCR control is shown. The relative mRNA expressions were obtained after normalizing the cycle threshold (CT) values from each gene with the internal PCR control, then using the ΔCT values from ES cells as a reference. All quantitative results shown are the mean of fold change (x) from 2 independent experiments, each performed in duplicate.
able to detect the expression of Oct-3/4, Nanog (Figure 1F), and Rex-1 by QRT-PCR in these cells directly isolated from Lin/CD45/CD31− BMMNCs and bulk mesenchymal cultures, although at a lower level compared with ES cells. We could not detect expression of these transcription factors in SSEA-1− cells. Interestingly, the levels of Oct-3/4 and Nanog expression were higher in freshly isolated cells than cultured SSEA-1− cells, suggesting that the simple culture medium employed in our studies was not suitable to maintain the expression of these transcription factors.

**SSEA-1+ cells at the apex of bone marrow mesenchymal compartment hierarchy**

Based on the fact that SSEA-1 expression was also seen on all the primitive RS1 cells, we hypothesized that SSEA-1+ cells are at the top of the mesenchymal stromal cell hierarchy. To prove this, we first sorted both SSEA-1+ (>90% purity) and SSEA-1− (>98% purity) fractions from MStrCs and placed them back into culture. Results clearly showed that after culture the SSEA-1− subpopulation gave rise to the negative fraction (Figure 2Ai−ii), whereas the reverse situation did not occur (Figure 2Aiii). This strongly suggests a differentiation and/or maturation from the SSEA-1+ to the SSEA-1− phenotype. Additionally we were able to verify that the SSEA-1+/KDR− fraction could give rise to both SSEA-1+/KDR+ (RS1) cells and SSEA-1− cells (Figure 2B). These hierarchical studies strongly suggest that SSEA-1 marks primitive cells from MStrCs and consequently we used SSEA-1 as our positive marker.

Secondly, we went on to demonstrate that these cells were also essential to establish adherent cell cultures. We highly enriched SSEA-1+ and SSEA-1− cells directly from Lin/CD45/CD31− or CD45/Ter119-depleted BMMNCs and put them back separately in culture with hematopoietic cells (as the putative mesenchymal fraction directly isolated from bone marrow requires certain factors and/or direct contact with hematopoietic cells to initiate adherent cultures). Only when SSEA-1− cells were used were mesenchymal cultures able to survive and propagate (Figure 2Civ). On the contrary, when we used the SSEA-1− fraction, most cultures either did not harbor any adherent cells or did not go beyond passage 0, as shown by the lack of the mesenchymal compartment (CD45/CD11b− fraction; Figure 2Civ).13 Thus, the presence of SSEA-1+ cells is essential to establish mesenchymal cultures and also to maintain the composition of these cultures in early cell passages, as they generate the SSEA-1− mesenchymal fraction.

Finally, to demonstrate that SSEA-1+ cells are indeed very primitive cells, we determined their in vitro differentiation capacity. For this experiment we compared enriched SSEA-1+ cells to SSEA-1− cells. SSEA-1+−enriched cells clearly showed higher expression of a panel of different antigens for each specific lineage (to osteoblast/osteocyte-, myoblast-, endothelial-, hepatocyte- and astrocyte-like cells) compared with the SSEA-1− fraction (Figure 3). The expression of these antigens was not detected in unstimulated cells from either fraction.

Also, the mesenchymal origin of the SSEA-1+ cells (both directly isolated from bone marrow and bulk cultures) was verified by positive staining for conventional mesenchymal markers such as CD105, CD73, Sca-1, CD44, fibronectin, and vimentin (data not shown).

**Characterization of the clonally derived SSEA-1+ population**

In order to address the multipotency of the SSEA-1+ cells, and since we showed that this population is heterogeneous, we performed clonality studies. Due to the peculiar nature of murine MStrCs, it was not possible to isolate/expand any putative mesenchymal cell type directly from the bone marrow (cells defined as Lin/CD45/CD31−) using culture conditions described here. To minimize the ex vivo expansion time required, single SSEA-1+ cells were isolated from passage 1–adherent cultures (from CD45/CD11b− fraction) and then expanded under MAPC culture conditions. We used the MAPC system based on the fact that factors contained in this conditioned medium were able to maintain the phenotype and features of SSEA-1+ mesenchymal cells without altering their in vitro multipotency (Figure S3). Preliminary studies using low cell doublings revealed that all clones had similar cell-expansion kinetics and cell-surface phenotype (Figure S4). However, the 3 clonally derived populations showed different in vitro differentiation potentials (Figure S5). Based on the fact that clone-3 cells demonstrated a broader differentiation capacity in vitro (Figure S5) and showed substantial expression of all 3 of the embryonic transcription factors tested here, this clone was selected for a more detailed characterization. Clone-3-derived cells were able to expand linearly over time with the maintenance of expression of SSEA-1, major histocompatibility complex class I.

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**Figure 2.** SSEA-1+ cells are at the apex of the murine mesenchymal compartment. (A) Purified SSEA-1+ (top left plot) and SSEA-1− (bottom left plot) cells were plated and analyzed a few days later. Analysis of SSEA-1 expression on SSEA-1− cultures after attaining half-confluency (i, n = 2) or confluency (ii, n = 3) and on SSEA-1+ cultures at confluency (iii, n = 3). (B) Schematic representation of the hierarchical organization of SSEA-1+ cells inferred from our studies. (C) Both enriched SSEA-1+ (R1) and SSEA-1− (R2) fractions were first purified from CD45/Ter119-depleted BMMNCs and then cultured independently in the presence of hematopoietic (R3) cells (i). (ii−iii) Overlay FACS plots show the purity of each enriched fraction (black dots) with the supporting hematopoietic cells (gray dots) as initiating culture populations. (iv) Representative FACS plots of adherent culture initiated by enriched SSEA-1+ cells (R2-derived cultures; n = 5) showing the lack of the putative mesenchymal fraction (CD45/CD11b−) at passage 0. In contrast, cultures initiated with SSEA-1− cells (R1-derived cultures; n = 3) were able to propagate and the presence of these cells was detected in different cell passages.
cells as shown by FACS analysis, immunostaining, and RT-PCR (Figure S6). Furthermore, these differentiated cells also acquired lineage-specific functional features7,14,16,21-24 (Figure S6).

Clone-3 SSEA-1+ cells can contribute to different mesenchymal cell types and endothelial- and hematopoietic-like cells in vivo

In order to evaluate the multipotency capacity of SSEA-1+ cells in vivo, we performed 2 sets of experiments. Prior to this, we verified that the expanded clone-3 population had a normal karyotype (Figure 4C; Table 1). The first set consisted of injecting unmanipulated clone-3 cells into nonconditioned NOD/SCID/β² null newborn mice and this was performed in 2 independent experiments. We used newborn mice on the basis that their tissues are at a fast-growing stage, hoping that it might create a better environment for incoming progenitor/stem cells to be incorporated and proliferate in adult mice. Tissue screening revealed frequent presence of MHC-I+/VWF+ endothelial cells in the liver (Figure S7). With the aim to find different donor-derived mesenchymal cell types, we mainly looked for the presence of MHC-I+ cells in the recipients’ bone marrows. To our surprise, we detected that most of the donor-derived cells were positive for the CD45 antigen (Figure 5; Table 2). Indeed, clonally derived SSEA-1+ cells were able to give rise to cells with a hematopoietic phenotype in all the recipient animals. Most MHC-I+/CD45+ cells expressed the myeloid marker Gr-1, although low levels of CD45/CD19+ and Ter119+ cells could also be detected (Figure 5E-F). Interestingly, multicolor flow-cytometry analysis revealed a high frequency of donor-derived cells with the CD45+/Lin-/c-Kit+‘Sca-1-’ (KLS) phenotype (Figure 5G-H).

We investigated whether these SSEA-1+–derived CD45/KLS cells had stem-cell properties. BMMNCs from engrafted mice were

Figure 3. Higher in vitro differentiation capacity of SSEA-1+. Comparative studies of in vitro differentiation capacity (n = 3–5) of SSEA-1+ and enriched SSEA-1+ populations (60%-70% purity). FACS analysis shows the level of specific antigen expression for each lineage tested: osteogenic (osteocalcin and bone sialoprotein II [BSP II]), myogenic (dystrophin and desmin), astrocyte (GFAP), hepatocyte (albumin and HNF-1), and endothelial (extracellular CD31 and VWF). Gray and white areas are the isotype control and specific antibody staining, respectively. The numbers shown indicate the levels of expression for each antigen that were calculated using the mean fluorescence intensity (MFI) values obtained from each specific staining over the values from their respective isotype controls. (A) Multicolor immunostaining performed on clone-3 cells at 45 doublings showing nuclear expression of Nanog, Rex-1, and Oct-4. Immunostainings were performed using Alexa 488– and Alexa 594–conjugated secondary antibodies. Appropriate rabbit IgGs and mouse IgG1 isotype controls were also used and cells were counterstained with DAPI. (B) Representative diploid metaphase spread of clone-3 population. Original magnifications, 40×/0.75 NA dry objective (C) and 100×/0.4 NA oil objective (D).

Table 1. Cytogenetic analysis of an adherent culture passage 1 (MCP1) and clone-3 SSEA-1+ cells at 48 doublings

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Both groups had normal karyotypes. chr indicates chromosomes.

(MHC-I), and CD44 and low levels of KDR and Trk at different time points (Figure S4). The abundant Oct-3/4, Rex-1, and Nanog mRNA transcripts detected in these cells (the fold differences in comparison to ES cells were $-56.8 \times$ for Oct-3/4, $-3.9 \times$ for Nanog, and $-17.9 \times$ for Rex-1) led us to examine their expression at the protein level. Indeed, the protein expression was also substantial and comparable to ES cells that were withdrawn from leukemia inhibitory factor (LIF) for 4 days (Figure 4A). We confirmed the nuclear localization of the above transcription factors using immunocytochemistry staining (Figure 4B). Again, the SSEA-1+ fraction was negative for these factors, further confirming the specificity of the results presented.

Also, clone-3 SSEA-1+ cells were able to differentiate into osteoblast/osteocyte-, endothelial-, hepatocyte-, and astrocyte-like...
We detected frequent GFP/endomucin$^+$ cells incorporated into the vascular canals that pass through the bone (Figure 6H–I), whereas some donor-derived endothelial cells could also be found in the marrow’s blood vessels (Figure 6G). To our surprise, with as few as 30,000 cells per inoculum, SSEA-1$^+$ cells were capable of not only highly differentiating into osteoblasts but also organizing themselves and incorporating extensively as bone-lining cells (Figure 6J). This was confirmed by the expression of osteocalcin (Figure 6K-M) and alkaline phosphatase (data not shown). Furthermore, abundant GFP$^+$ cells were seen incorporated in the bone, mostly expressing osteocalcin or BSP II, confirming their osteocytic nature (Figure 6N-P). Occasional single GFP$^+$ adipocytes were observed in most of the animals, with the exception of 1 mouse in which abundant donor-derived adipocytes were found (Figure 6T). This same mouse also showed a different pattern of engraftment in comparison to the others. Patches of concentrated GFP$^+$ cells were found with incorrect deposition of type II collagen (Figure 6S), indicating formation of cartilaginous tissue in the marrow cavity. The reason for this is unknown. We can only speculate that the cells were “clumped” together when they were inoculated and, due to their high “plastic” properties, clumping might have stimulated the cells to differentiate into adipocytes and chondroblasts (and subsequently to cartilage formation) instead of osteoblasts/osteocytes. Thus, not only the cell number but also the location and the state in which the cells are administered could determine the outcome of differentiation. This has important safety implications in regard to the potential use of these cells in cell-therapy settings.

Gene-expression profile of SSEA-1$^+$ cells

The surprising contribution of SSEA-1$^+$ mesenchymal cells to other mesodermal lineages other than mesenchymal cells in vivo led us to examine the expression of different mesoderm-associated genes by QRT-PCR (Figure 7A). We determined the gene profile of the 3 clonally derived populations, bulk SSEA-1$^+$ cells, and CD45/CD11b/Ter119/CD31$^-$ SSEA-1$^+$ and SSEA-1$^-$ fractions directly isolated from the bone marrow. As half of the populations were not clonally derived, we thought that it was not appropriate to compare the expression of each gene between the different populations. Instead, we wanted to obtain a pattern of gene expression for each population. As such, we compared the selected genes among them, using the Brachyury (T) gene as reference (Figure 7B). The expression data revealed that all cultured and noncultured SSEA-1$^+$ cells express Brachyury (T), PDGF-Ra, and Gata-2, although at different levels. Meanwhile, the CD45/CD11b/Ter119/CD31$^-$ SSEA-1$^-$ bone marrow–derived fraction showed only the expression of Scl/Tal1 and Runx1. As most of the populations showed high levels of Gata-2 (exon 5/6) mRNA

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<td>86.5</td>
<td>0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The percentages of MHC-1$^+$ cells represent the number of positive cells found over the total BMNCs analyzed, whereas the other values represent their respective percentage over the MHC-1$^+$ cells. ND indicates not determined.
transcripts, we investigated whether these populations express the hematopoietic stem/progenitor-specific isoform (1S).27,28 To our surprise, clone-3 and noncultured SSEA-1+ cells express the primary transcript for this specific isoform (1S exon/1S intron), albeit at lower levels than KLS cells. These findings suggest that at least at the mRNA level this isoform is not confined only to hematopoietic stem/progenitor cells.

In general, noncultured SSEA-1+ cells have a broader expression of mesoderm-associated genes, whereas the other populations have a more confined pattern. E-cadherin and Sox17 (endoderm-associated genes)26,29 were also detected in this fraction but no Sox1, Sox3, or Oligo2 expressions were found (data not shown). The data show that Runx1, VE-Cad, Scl/Tal1, and KDR expression levels are the highest followed by PDGF-Rα and Gata-2 in noncultured SSEA-1+ cells. Even though the SSEA-1+ mesenchymal cells in the bone marrow are a heterogeneous population and therefore might include some cells with some features of endoderm cells, the overall pattern of this population shares more similarities with mesodermal progenitors.24-26,29

Interestingly, clone-3 cells express genes for both lateral and paraxial mesoderm.24,26 The high expression of Runx1, Gata-2, KDR, VE-Cad, c-Kit, and PDGF-Rα observed might reflect the fate of this population as described here.

**Discussion**

Most of the understanding of bone marrow–derived mesenchymal cells came from in vitro studies using mixed populations of adherent cells that might contain a putative stem cell. However, this putative mesenchymal stem cell is yet to be identified and isolated. Although in recent years, work from Prockop (Colter et al2-3), Simmons (Gronthos et al6-7), and Verfaillie (Jiang et al9,10) has contributed greatly to our understanding of these cells, the lack of an appropriate marker for identifying/isolating the primitive subset from bone marrow and/or the adherent cultures has been the main drawback for the much-desired progress in this area. In light of past findings, we wished to unravel the hierarchical structure of the adherent cell cultures derived from adult mouse bone marrow.

We report the existence of a minor subpopulation in the adult bone marrow–derived mesenchymal cells that expresses the embryonic
cells to survive ex vivo, we were unable to assess the in vitro differentiation potential of uncultured SSEA-1+ cells. As such, we cannot rule out that the high differentiation capacity of SSEA-1+ cells from bulk cultures could have resulted from ex vivo conditions. However, based on their higher expression of Oct-3/4 and Nanog, we believe that the cells from bone marrow should behave similarly to their cultured counterparts. Furthermore, the gene-profile data suggest that uncultured cells might even represent a more primitive population.

One of the most interesting findings in this study is the observation that SSEA-1+ mesenchymal cells were able to contribute to other mesodermal lineages in vivo, including hematopoietic cells. Indeed, we were able to detect hematopoietic-like contribution in 2 independent experiments using newborn mice and also in conditioned adult mice. As we were not able to reproduce engraftment in secondary recipients, further studies will be needed to ascertain the long-term repopulating capacity of the hematopoietic cells generated or to determine whether this was only a transient reconstitution. Clearly, we cannot totally rule out the possibility that SSEA-1+ mesenchymal cells had fused with donor hematopoietic stem cells (HSCs)/hematopoietic cells, as we have not designed our experiments to address this issue. Nonetheless, we showed that SSEA-1+ mesenchymal cells could efficiently differentiate into endothelial cells, suggesting that these cells can contribute to cell types other than mesenchymal cells in vivo. Also, our gene-expression data show that the clone-3 population expressed genes for both lateral and paraxial mesoderm lineages, suggesting that these cells might have the potential to differentiate into not only mesenchymal cells but also endothelial and hematopoietic cells. Indeed, clone-1 and clone-2 populations (with a more-confined pattern of gene expression) were unable to give rise to hematopoietic-like cells in vivo (data not shown).

Altogether, these findings indicate that the SSEA-1+ mesenchymal population may not represent mesenchymal progenitors but rather postnatal mesodermal progenitors. In fact, accumulating evidence suggests that a common precursor of mesenchymal and hematopoietic lineages might exist.30-33 We are in the process of investigating further the true nature of these cells by injecting them into early blastocysts and assessing their contribution into different tissues.

In summary, our study shows the identification of a novel multipotent cell population that resides in the bone marrow. Although further investigation is still required to better understand the biology of these cells, we hope that our study will provide the foundation for future research into the hierarchy of the mesenchymal lineage and the developmental origin of these cells with respect to endothelial and hematopoietic cells.

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Authorship

Contribution: F.A.-A. designed research, performed research, analyzed data, and wrote the paper; and D.B. designed research, contributed reagents and analytical tools, and wrote the paper.

References

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Nonhematopoietic/endothelial SSEA-1$^+$ cells define the most primitive progenitors in the adult murine bone marrow mesenchymal compartment

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