Molecular profile of mouse stromal mesenchymal stem cells

Sebastien Chateauvieux, Jean-Laurent Ichante, Bruno Delorme, Vincent Frouin, Genevieve Piétu, Alain Langonné, Nathalie Gallay, Luc Sensebé, Michèle T. Martin, Kateri A. Moore, and Pierre Charbord

1Institut National de la Santé et de la Recherche Médicale, Equipe-ESPRI/EA-3855, Université François Rabelais, Faculté de Médecine, Tours; 2Commissariat à l’Energie Atomique, Service de Génomique Fonctionnelle, Evry; and 3Etablissement Français du Sang-Centre-Atlantique, Tours, France; and 4Department of Molecular Biology, Lewis Thomas Laboratory, Princeton University, Princeton, New Jersey

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DURING DEVELOPMENT, HEMATOPOIESIS takes place in sequential sites (reviewed in Ref. 16). It develops during embryonic life within the extraembryonic annex, the yolk sac, and within the intraembryonic region, the aorta-gonad-mesonephros (AGM) region. During fetal life, it proceeds within the liver. From birth onward, bone marrow is the major, if not exclusive, site of hematopoiesis in mammals. Hematopoietic stem cells (HSCs) migrate, via the bloodstream, from one site to the next. As early as the AGM stage, HSCs have acquired their essential properties of self-renewal and multipotentiality, giving rise to all blood lineages. It is in the fetal liver that HSCs show maximal proliferation capacity: only at this stage is amplification of the HSC pool observed.

Cells from the microenvironment provide different niches for hematopoietic cells. In the HSC niche (reviewed in Refs. 1, 24, 30, 48), the stemness, i.e., the balance between self-renewal and commitment, is insured by physical contact between the HSC and a “stromal” cell (i.e., hematopoietic supportive) and its associated extracellular matrix. In the bone marrow, many recent experiments strongly suggest that the stromal component would be an osteoblastic-type cell or a highly specialized microvascular cell (1). In the fetal liver, fewer studies have tried to define the stromal component of the niche. We have suggested (7) that a cell in epithelial-to-mesenchymal transition would constitute such a stromal component; the final adult fate of such a cell would be hepatocytes and sinusoid vascular smooth muscle cells that would no longer possess the stromal capacity.

In all major hematopoietic sites (AGM, fetal liver, and bone marrow), a new type of stem cells has been described: the mesenchymal stem cells (MSCs) (6, 29, 36). MSCs are multipotential cells that differentiate into adipocytes (A), osteoblasts (O), chondrocytes (C), and vascular smooth muscle cells (V) (reviewed in Refs. 5, 13). It has been suggested that MSCs are identical to stromal cells used as feeder layers for long-term cultures of HSCs (37). In this work, we searched for mesenchymal clonal lines with A, O, C, and V differentiation potential and with similar stromal capacity. We found that two lines had such potential, one being from adult bone marrow, BMC9, and the other from the fetal liver, AFT024. We then tried to establish the transcriptomic profile specific for stromal MSCs independent of the hematopoietic site. To this end, we ascertained the set of genes differentially expressed in the intersection set of AFT024 and BMC9 compared with those expressed in the union set of two negative control lines, 2018 and BFC012 (both from fetal liver); 346 genes were upregulated and 299 downregulated. Using Ingenuity software, we found two major gene networks with highly significant scores. One network contained upregulated genes that belonged to two categories, cytoskeletal genes and genes implicated in the transcriptional machinery. The data extend the concept of stromal mesenchymal stem cells from adult and fetal hematopoietic sites. To identify mesenchymal stem cell-like stromal cell lines, we evaluated the adipocytic, osteoblastic, chondrocytic, and vascular smooth muscle differentiation potential and also the hematopoietic supportive (stromal) capacity of six mouse stromal cell lines from adult bone marrow and day 14.5 fetal liver. We found that two lines were quadripotent and also supported hematopoiesis, BMC9 from bone marrow and AFT024 from fetal liver. We then ascertained the set of genes differentially expressed in the intersection set of AFT024 and BMC9 compared with those expressed in the union set of two negative control lines, 2018 and BFC012 (both from fetal liver); 346 genes were upregulated and 299 downregulated. Using Ingenuity software, we found two major gene networks with highly significant scores. One network contained upregulated genes that are known to be implicated in osteoblastic differentiation, proliferation, or transformation. The other network contained upregulated genes that belonged to two categories, cytoskeletal genes and genes implicated in the transcriptional machinery. The data extend the concept of stromal mesenchymal stem cells to clonal cell populations derived not only from bone marrow but also from fetal liver. The gene networks described should discriminate this cell type from other types of stem cells and help define the stem cell state.

MATERIALS AND METHODS

Cell Lines

Lines used in this work were either 14.5 days postcoitum (dpc) fetal liver lines (AFT024, 2012, 2018, and BFC012) provided by K.A. Moore or bone marrow lines (BMC9 and BMC10) kindly provided by James E. Dennis (Case Western Reserve University, Cleveland, OH). All lines were immortalized using SV-40 large T-antigen. Large temperature-sensitive T-antigen (TSa-58) lines were made by retroviral transduction into the fetal liver stroma and clonally derived; the
clonal nature was verified by Southern blot analysis, which detected a single, unique proviral integration locus in the genomic DNA. The bone marrow lines had been obtained from transgenic immunomice generated by embryonic stem (ES) cells transfected with thermosensitive T under the major histocompatibility complex promoter. The lines were developed from isolated clones. Fetal liver cell lines were cultured at 33°C in 5% CO₂ in high-glucose DMEM with 10% (vol/vol) screened fetal calf serum (FCS, Hyclone), 0.1% β-mercaptoethanol, 2 mM L-glutamine, and antibiotics. Bone marrow cell lines were cultured at 33°C in 5% CO₂ in α-MEM with 10% FCS, 100 IU/ml (50 ng/ml) γ-interferon, 2 mM L-glutamine, antibiotics, and antimiycotics.

Differentiation Studies

Osteoblastic differentiation was induced by culture in DMEM (high glucose) medium with 10% FCS, 0.1 μM dexamethasone, 2 mM β-glycerophosphate, and 150 μM ascorbate-2-phosphate. Cells were seeded at 10,000 cells/cm² and incubated for 21 days at 37°C. Medium was changed every 4 days.

Adipogenic differentiation was induced by culturing in DMEM (high glucose) medium with 20% FCS, 1 μM dexamethasone, 0.35 μM hydrocortisone, 0.5 mM isobutyl-methylxanthine (IBMX), 100 ng/ml insulin, and 60 μM indomethacin. Cells were seeded at 20,000 cells/cm² and incubated for 12 days at 37°C. Medium was changed every 4 days.

Chondrogenic differentiation was obtained in micropellets (3 – 10³ cells/pellet) incubated at 37°C for 21 days in 500 μl of chondrogenic medium composed of DMEM (high glucose) with 0.1 μM dexamethasone, 1 mM sodium pyruvate, 170 μM ascorbic acid-2-phosphate, 350 μM proline, 1 × insulin-transferrin-selenium, and 10 ng/ml transforming growth factor (TGF)-β1. Medium was changed every 4 days. RNA studies required the use of 20–30 micropellets.

Vascular smooth muscle differentiation was obtained in long-term culture medium: McCoy’s 5 A medium with 12.5% screened horse serum, 12.5% FCS, 20 μM L-glutamine, 0.8 mM L-serine, 0.15 mM L-asparagine, 1 mM sodium pyruvate, 5 mM sodium bicarbonate, 1 μM hydrocortisone, and antimiycotics and antibiotics. Cells were passaged at the end of the first week; during the two remaining weeks, medium was changed every 4 days.

Cytoskeletal staining. Alkaline phosphatase was evaluated, after 4 and 15 days of culture in osteogenic medium, with the Alkaline Phosphatase Assay Staining Kit (Bio-Rad). Cells were detached using phosphate-buffered saline (PBS) with 1 mM EDTA. Primary antibodies were purified rat anti-mouse mAbs from BD Biosciences: CD134 (RM4-5), CD44 (IM-7), CD45 (30F-11), CD49d (R1-2AKR), CD49e (5H10-27), CD105 (MJ7/18), CD106 (429), and Sca-1 (D7) or their negative controls (rat IgG1, IgG2a, and IgG2b). Secondary antibody was goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad). Revelation by chemiluminescence was performed by ECLPlus Western Blotting Detection Kit (Amersham Biosciences) and acquisition with Chemi-Smart 2000 using Chemocapt software (Vilbert-Lourmat). Flow cytometry. Cells were detached using phosphate-buffered saline (PBS) with 1 mM EDTA. Primary antibodies were purified rat anti-mouse mAbs from BD Biosciences: CD134 (RM4-5), CD44 (IM-7), CD45 (30F-11), CD49d (R1-2AKR), CD49e (5H10-27), CD105 (MJ7/18), CD106 (429), and Sca-1 (D7) or their negative controls (rat IgG1, IgG2a, and IgG2b). Secondary antibody was phycoerythrin (PE)-conjugated goat anti-rat antibody. Cells were passed through a FACSCalibur flow cytometer (Becton-Dickinson) using a 488-nm argon laser. Samples were analyzed by collecting 10,000 events using CellQuest software (Becton-Dickinson).

Hematopoietic Supportive (Stromal) Capacity

Cobblestone area-forming cell. The hematopoietic population of precursors was purified from 4- to 6-wk-old C57Bl/6J mouse cells. ScAl⁻/Lin⁻ cells were isolated following, first, depletion of lineage-positive cells using a lineage cell depletion kit (Miltenyi) and, second, isolation of ScAl⁻/Lin⁻ cells with a PE selection kit (EasySep) using anti-ScAl antibody conjugated to PE (D7, BD Biosciences). The protocol for the study was submitted to and approved by the local Ethical Committee. The purity was >95%, as evaluated by flow cytometry. ScAl⁻/Lin⁻ cells were plated on irradiated (15 Gy) confluent layers of AFT024 or BMC9 grown on 96-well plates, using 6–24 replicas of 7 cell concentrations (1–200 cells/well). Cobblestone area-forming cell (CAFC) frequency was determined by limit-dilution analysis using Poisson statistics.

Flow cytometry. Analysis of hematopoietic precursors co-cultivated for 5 wk with the stromal layers was carried out using flow cytometry. Cells were labeled with rat anti-mouse mAbs from BD Biosciences: anti-CD45 (30-F11) conjugated to allophycocyanin (APC), anti-Sca-1 (D7) conjugated to PE, and antibodies conjugated to FITC, either CD4 (GK1.5) + CD8α (53-6.7) + CD45R (B220), or CD11b (M1/70), CD51 (RMV-7), CD14 (55-3), and Ter-119 (Ter-119).

Gene Screening by CEA Chip

The mouse CEA chip consists of 13,060 cDNA clones from early mouse embryo libraries constructed by the National Institute on Aging (NIA; http://www.nia.nih.gov) representing ~11,000 genes. It also
includes positive [actin, tubulin, oligo(dT), cot-1 DNA] and negative (Tris-EDTA/DMSO) controls present many times in the array for quality control. Microarrays were prepared by spotting PCR products as previously described. The DNA chip used for this experiment is described and available at Gene Expression Omnibus (GEO; GPL3438 and GPL3439).

RNA extraction. For each line, RNA was extracted from two independent cultures. Total RNA was isolated using the Trizol reagent (Invitrogen) and dissolved in RNase-free water. The quality of RNA was controlled using an Agilent 2100 Bioanalyzer (Agilent Technologies), and concentration was measured by absorbance at 260 nm. For each target preparation, 20 μg of total RNA were reverse transcribed using Superscript II RT (Life Technologies) in the presence of an oligo(dT)-primed reaction and an amino-modified nucleotide (aminomethyl-dUTP). Amino-modified cDNAs were purified through a Microcon Centricron 30 microconcentrator (Amicon) and ethanol precipitated. In a second step, monofunctional forms of Cy3 and Cy5 dyes were coupled to the purified amino-modified cDNAs. Unincorporated fluorescent molecules and salts were removed through the Microcon Centricron 30. Labeled cDNA was mixed with 10 μg of poly(A) RNA (Boehringer), 10 μg of tRNA (Life Technologies), and 10 μg of mouse Cot1 DNA (Life Technologies). Purified labeled targets were hybridized to the array overnight as previously described (3). The cell lines 2018, BFC012, and BMC9 were each compared with AFT024. Each comparison was performed four times in both labeling orientations (8 hybridizations per comparison, 4 dye swaps).

Microarray data processing. Fluorescence intensities of Cy3 and Cy5 were measured separately at 532 and 635 nm, respectively, with a laser scanner (Axon GenePix 4000A). Image analysis was performed with GenePix Pro 4.0 software (Axon). Spots with obvious blemishes were flagged. Genes with null intensity values and low mean intensity were also annotated with a specific flag and excluded from the list of validated differentially expressed genes. Statistical analysis of experimental data was performed after an intensity-dependent LOWESS normalization (47) with BioConductor/Limma (version 1.8.16) (39), using a standard ANOVA performed on the logarithm of gene expression data without background subtraction as described in Ref. 12. We did not apply a threshold on the ratio values of gene expression but instead used the t-test to determine significance as such: results significantly different from 1 (P value < 0.05) were determined from the eight values obtained for each comparison. The microarray data set used for this analysis is described and available from GEO (GSE4491).

Gene network analysis. The interactions between genes identified as differentially expressed and all other genes were investigated using the Ingenuity Pathways Analysis (IPA). A series of networks was generated; a network is defined by IPA as the reflection of all interactions of a given protein defined in the literature. The interaction indicates physical association or consists of induction/activation or repression/inactivation/degradation of one gene product by the other, eventually through another intermediary molecule (indirect interaction). The program calculates a statistical score based on a right-tailed Fisher’s exact test that calculates the fit of each network to the set of studied genes. A score >2 indicates that there is a less than 1 in 100 chance that the studied genes are assembled into the network because of random chance. We considered only the networks with the highest scores (>50). We introduced in the analysis two sets: that of upregulated genes and that of downregulated genes. The two major networks for up- and downregulated genes were then compared to find out the interactions among genes belonging to either network.

RESULTS

Previous studies have shown that the four 14.5 dpc fetal liver lines (AFT024, 2012, 2018, and BFC012) varied greatly in their ability to support hematopoiesis, with AFT024 being the best supporter (18). The mesenchymal differentiation potential of the fetal liver lines was not evaluated in previous works. On the contrary, in another report, the adult bone marrow line BMC9 was shown to be tripotent (with A, O, and C differentiation potential) and supportive for osteoclasts, while BMC10 was deficient in both A differentiation and osteoclast support (14).

AFT024 and BMC9 Are Quadrupotent Mesenchymal Lines

After 12 days in adipogenic medium, many AFT024 and BMC9 cells contained large (~5 μm in diameter) NRO+ vesicles (Fig. 1A), and adipocytic markers (Fig. 2A) were induced (lipoprotein lipase; Lpl) or increased [fatty acid-binding protein (Fapb4) and peroxisome proliferator-activated receptor-γ (Pparg)]. After 21 days in osteogenic medium, many von Kossa+ mineralized areas were apparent (Fig. 1A), and for AFT024, the osteocalcin (Bglap) osteoblastic marker was increased (Fig. 2A). Alkaline phosphatase was significantly increased in both lines at day 4 (P < 0.05) and day 15 (P < 0.001) (Fig. 2B). After 21 days in chondrogenic culture conditions, safranin O+ well-formed micropellets were apparent (Fig. 1A), and the aggrecan-1 (Agc1) chondrocytic marker was clearly induced in both lines (Fig. 2A). After 21 days of cells being in long-term culture medium inducing V differentiation, immunofluorescence studies indicated the presence of microfilaments containing smooth muscle myosin in most AFT024 cells and about one-half of BMC9 cells (Fig. 1B). α-Smooth muscle actin (Acta2) transcripts in AFT024 and desmin (Des) transcripts in both lines were slightly increased (Fig. 2A). Western blotting studies indicated that the smooth muscle myosin heavy chain SM1 was enhanced in AFT024, h-caldesmon was clearly induced in both lines, and metavinculin was detected in both (Fig. 2C). Finally, all cells from both lines were expressed, at high a level, CD106, Sca-1, and CD49e; on the other hand, there was no expression of CD45, CD34, CD49d, and CD90 (not shown).

In 2012 and BMC10 cells, after A differentiation, most NRO+ vesicles (Fig. 1A) were of small size, and Fapb4 and Pparg were enhanced, while Lpl was not detected in BMC10 (Fig. 1B). After O differentiation, many von Kossa+ mineralized areas were apparent (Fig. 1A), and Bglap was increased (Fig. 2A). Alkaline phosphatase was significantly increased at day 4 (P < 0.001) in 2012 and day 15 (P < 0.001) in both lines (Fig. 2B). After C differentiation, micropellets contained few safranin O+ areas (Fig. 1A). After V differentiation, Acta2 and Des transcripts remained unchanged (but the band was faint in 2012) (Fig. 2A); h-caldesmon, SM1, and metavinculin were increased in 2012, but h-caldesmon and SM1 remained barely detectable in BMC10 (Fig. 2C). The immune phenotype was as described for AFT024 and BMC9 (not shown).

In 2018 and BFC012, after A differentiation, few cells with small-sized NRO+ vesicles were detected (Fig. 1A); in 2018, but not BFC012, Lpl, Fapb4, and Pparg were induced (Fig. 2A). After O differentiation, von Kossa+ mineralized areas were not detected in either lines (Fig. 1A), but in 2018, Bglap was detected before differentiation and unchanged after (Fig. 2A). Alkaline phosphatase was significantly increased at day 4 (P < 0.001) in 2018 and day 15 (P < 0.001) in both lines (Fig. 2B), which suggested that both lines were able to differentiate, more or less readily, into O but were defective.
for mineralization. After C differentiation, micropellets contained no safranin O areas (Fig. 1A), and Agc1 induction was weak in BFC012 (Fig. 2A). After V differentiation, Des transcripts were induced in 2018, but barely in BFC012, and Acta2 was unchanged (Fig. 2A); a faint h-caldesmon band was apparent, but no metavinculin, while SM1 was unchanged in BFC012 (Fig. 2C). CD49e expression was low in both lines; Sca-1 was not detected in BFC012, and CD34 was expressed in both lines (not shown). 

In conclusion, AFT024 and BMC9 were clearly quadripotent and showed an immune phenotype of murine MSCs. All other lines showed impaired differentiation at one or several levels. The lines with the most impaired differentiation potential were 2018 and BFC012; these lines also showed unusual expression of some of the membrane markers.

**AFT024 and BMC9 Are Stromal Lines**

The hematopoietic supportive ability was studied in both lines by seeding irradiated cells with bone marrow mouse Sca1/H11001/Lin/H11002 cells (Fig. 3). The CAFC frequency (Fig. 3, C and D) from two HSC enrichments after 5 wk was 1/40–1/50 and 1/110–1/160 for AFT024 and BCM9, respectively. Flow cytometry studies (Fig. 3B) indicated the persistence of immature
Sca1+/CD45+ hematopoietic cells along with more mature CD11b+/CD45+ granulocytes, CD14+/CD45+ monocytes, and CD51+/CD45+ megakaryocytes, without significant difference between the two cell lines. Very few TER119+/CD45+ erythroid cells were detected on BMC9. CD4+/CD45+, CD8+/CD45+, or CD45R+/CD45+ lymphoid cells were not detected. The presence of megakaryocytes, polymorphs, and monocytes was confirmed by cytology after May Grunwald Giemsa staining (Fig. 3A). In conclusion, both lines displayed hematopoietic support, with an advantage for AFT024 in terms of CFU frequency.

**Molecular Profile of Stromal MSCs**

Results from the above cellular studies indicated that AFT024 and BMC9 were adequate models for stromal multipotential MSCs, whereas 2018 and BFC012 with severely defective stromal and differentiation potential were negative control lines. To determine the molecular profile specific for stromal multipotential MSCs independent of the hematopoietic site, we ascertained the set of genes differentially expressed in the intersection set of AFT024 and BMC9 compared with those expressed in the union set of the two negative control lines 2018 and BFC012. The lines BMC10 and 2012, with partially defective differentiation and stromal potential, were not considered in the analysis. Our strategy was, first, to determine the set of genes included in the union of genes expressed by 2018 and BFC012 (U1); second, to subtract this union set from genes expressed by AFT024 (S1) and by BMC9 (S2); and, third, to determine the intersection of S1 and S2 (S3). There were 346 upregulated and 299 downregulated genes in S3. Functional classification is shown in Fig. 4. Hierarchical clustering validated our strategy, the genes up- or downregulated in BMC9 (2 distinct extractions, 8 dye swaps) showing the greatest similarity to those in AFT024, whereas those in BFC012 showed the greatest dissimilarity (Fig. 5).
Supplemental Table 1 (supplemental data are available at the online version of this article) gives the list of downregulated genes belonging to the following categories: cytoskeleton, transcription and chromatin remodeling, signaling, adhesion, trafficking, metabolism, cell cycle and apoptosis, protein synthesis and degradation, and other. Study of gene networks using Ingenuity software revealed a major network of 35 genes, with a highly significant score of 64 (Network 1, Fig. 6A). Ten of the downregulated genes in this network coded for proteins implicated positively in osteogenic differentiation, proliferation, or transformation: protooncogene protein c-fos (Fos), parathyroid hormone-related protein (Pthlh), adrenomedullin (Adm), receptor activity-modifying protein 2 (Ramp2), apoptosis regulator Bcl-2 (Bcl2), ezrin (Vil2), estrogen receptor 1 (Esr1), proline-rich nuclear receptor co-activator 1 (Pnrc1), guanine nucleotide-binding protein G, α-subunit (Gnas), and regulator of G protein signaling 2 (Rgs2).

Supplemental Table 2 lists the upregulated genes grouped into the same categories as in Supplemental Table 1. Study of gene networks using the Ingenuity software revealed a major network of 35 genes, with a highly significant score of 56 (Network 2, Fig. 6B). Most interactions in this network were direct and consisted of physical association or induction. Transcripts belonged to two categories, transcripts for cytoskeletal molecules and transcripts implicated in the regulation of the transcriptional machinery (including chromatin remodeling). The genes of this network encoding proteins implicated in the cytoskeleton were as follows: the structural proteins tropomyosins (Tpm3, Tpm1), moesin (Msn), cytoplasmic 1 actin (Actb), cytoplasmic 2 actin (Actg1), the molecular chaperones for actin and tubulin, T-complex protein 1 subunit-α (Cct3), -β (Cct5), and -γ (Cct7), and molecules involved in the regulation of microfilament or microtubule assembly/dynamics, rhophilin (Rhpn2), transforming protein RhoA (Rhoa), phospholipase D1 (Pld1), myosin regulatory light chain MRCL2 (Mrcl2), protein diaphanous homolog 3 (Diaph3), and ADP-ribosylation factor 1 (Arf1). Gene Ontology analysis confirmed the significance (P < 0.02 by Fisher’s exact test) of the overrepresentation of biological processes, cellular components, and molecular functions involved in cytoskeletal organization. The complete list of upregulated genes (Supplemental Table 2) confirmed the presence of many cytoskeletal genes not included in the network described above. The molecules of the network involved in the regulation of transcription were as
follows: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin (Smarcc1), the splicing factors heterogeneous nuclear ribonucleoprotein-A1 (Hnrpa1), -D0 (Hnrpd), -U (Hnrpu) and -K (Hnrpk), the arginine/serine-rich proteins 1 and 3 (Sfrs1, Sfrs3), ruvB-like 1 (Ruvbl1), actin-like protein 6A (Actl6a), putative RNA-binding protein 3 (Rbm3), GATA-binding protein 2 (Gata2), four and a half LIM domains protein 2 (Fhl2), zinc finger protein 638 (Znf638), high-mobility group nucleosome-binding domain-containing protein 2 (Hmgn2), protein flightless-1 homolog (Flii), and Cullin-1 (Cul1). Some of the cytoskeletal molecules are known to be associated to molecules involved in the transcription machinery: Rhoa is functionally associated to Fhl2, as activated Rhoa induces the translocation of Fhl2 to the nucleus and its subsequent activation (31); Actl6a, Ruvbl1, and Smarcc1 are part of molecular complexes associating transcriptional regulators to molecules of the cytoskeleton such as Actb (15, 34). Two transcripts, Fhl2 and Ruvbl1, are implicated in the Wnt signaling pathway, known to modulate the transcriptional transactivation of β-catenin in association with LEF1/TEF factors (45).

Certain molecules of the network of downregulated genes (Network 1) are known to be physically associated to those of

Fig. 4. Functional classification of genes up- and downregulated in mesenchymal stem cells (MSCs). This classification was performed after analysis of the data in the literature for each of the genes.

Fig. 5. Hierarchical clustering of genes up- and downregulated in MSCs. Each column indicates the level of gene expression in BMC9, 2018, and BFC012, as related to that in AFT024. Color scale indicates the degree of similarity. Gene clusters are shown at left and right sides (for upregulated and downregulated genes, respectively). The Sr-2 red box includes 11 genes present in the network of upregulated genes (shown in Fig. 6B).
the network of upregulated genes (Network 2) (Fig. 6): villin 2 (Network 1) to moesin and cytoplasmic 1 actin (Network 2); and NF-kB inhibitor-α and Fos (Network 1) to different heterogeneous nuclear ribonucleoproteins and to cytoplasmic 2 actin, ADP-ribosylation factor 1, and RhoA (Network 2) (2, 10, 11, 17, 19, 22, 32, 44). Eleven (of 35) molecules of the network of upregulated genes were localized in a specific cluster, Sr-2, that contained 46 genes (Fig. 5).

The molecules included in the two networks and their interacting partners are listed in Supplemental Table 4.

DISCUSSION

Our study indicates that two mouse mesenchymal clonal lines, one from bone marrow (BMC9) and the other from fetal liver (AFT024), possess the characteristics of stromal MSCs. Both lines were able to differentiate into adipocytes, mineralizing osteoblasts, chondrocytes, and vascular smooth muscle cells and supported Sca1+/Lin− mouse bone marrow hematopoietic precursors, allowing the generation of cobblestone areas and the differentiation of myeloid precursors. On the contrary, in the other lines, the mesenchymal differentiation potential was impaired at one or several levels. The differentiation defect was most severe in 2018 and BFC012. These lines gave rise to few adipocytes, did not generate mineralizing osteoblasts, gave, under chondrogenic condition, loose aggregates that were safranin O negative, and expressed, after V differentiation, low amounts of h-caldesmon. In addition, it has been shown in a previous study that none of these two lines allowed the maintenance of HSCs (18).

It has been suggested that the two populations, that of MSCs (isolated by their adherence to plastic and containing stem cells/progenitors for the A, O, C lineages) and that of stromal cells (used as feeder layers for long-term cultures of HSCs), are identical (37). This hypothesis has been sustained for culture-expanded bone marrow primary human MSCs (28). Our study extends this concept of stromal MSCs to clonal cell populations derived not only from bone marrow but also from fetal liver.

To determine the transcriptomic profile specific for stromal multipotential MSCs independent of the hematopoietic site, we ascertained the set of genes differentially expressed in the intersection set of AFT024 and BMC9 compared with those expressed in the union set of the two negative control lines, 2018 and BFC012. We found that 645 transcripts were specifically regulated in the two stromal multipotential cell lines, with a similar number of up- and downregulated genes. Some of the upregulated transcripts appeared to be of special interest and already have been described in the literature on stromal cells: the serine/threonine kinase NLK (Nlk) and the Jumonji protein 1a and 2 (Jarid1a, Jarid2), because of their demonstrated role as stromal mediator and differentiating factor for stromal cells (23, 25), and the lymphocyte-specific adapter protein Lnk (Lnk), because of its implication in the interaction between HSCs and cells from the microenvironment (41).
differentiation using the micropellet technique, requiring large
sufficient proliferation, in particular when studying chondrogenic
lines) or for their differentiation potential (bone marrow lines).
previously (14, 18), either for their stromal capacity (fetal liver
reasons.

Using the micropellet technique, it is possible to immortalize
kines).
implicated in stromal support (8) are insufficiently represented
particular, some categories found in other genetic screens as
regulated genes might be larger than what is described here. In
the known mammalian genome, the number of up- and down-
confirm this hypothesis.

Studies using short interfering RNA (20, 40) should
49). Such transcripts may be characteristic of the stem cell state

As shown in Table 1, 42 transcripts in our set of upregulated
genes were also found upregulated in other embryonic or adult
hematopoietic or neural stem cells (4, 21, 27, 33, 38, 42, 43,
9). Such transcripts may be characteristic of the stem cell state
(50). Studies using short interfering RNA (20, 40) should
confirm this hypothesis.

Because our gene chip encompasses about only one-third of
the known mammalian genome, the number of up- and down-
regulated genes might be larger than what is described here. In
particular, some categories found in other genetic screens as
implicated in stromal support (8) are insufficiently represented
in the chip used here (especially proteoglycans and chemokines).

We chose to study specific clones that were immortalized
using a temperature-sensitive SV-40 T-antigen for the follow-
ing reasons. 1) These clones have been studied in detail
previously (14, 18), either for their stromal capacity (fetal liver
lines) or for their differentiation potential (bone marrow lines).
2) The introduction of the viral sequence would insure sufficient
proliferation, in particular when studying chondrogenic
differentiation using the micropellet technique, requiring large
cell numbers. 3) Mouse cells immortalized by T show a
phenotype comparable to nontransduced cells (9).

To assess the potential influence of viral genes on the
expression of cell genes, we have recently compared, using
microfluidic cards from Applied Biosystems, the expression of
380 transcripts in clones derived from primary cultures of
mouse bone marrow MSCs vs. BMC9 and BMC10. We have
found that 7% of the transcripts were up- or downregulated in
cells with large T. The studied transcripts belonged to several
categories. The least affected category was that of cytoskeletal
components (only 3% of the genes in this category were
significantly modulated). On the other hand, the most affected
category was that of molecules involved in cell cycle and
apoptosis: 39% of the studied cyclins, cyclin inhibitors, and
apoptosis inhibitors were upregulated in BMC9 and BMC10.
Molecules implicated in the transcriptional machinery were
only marginally affected (6%). These data indicate that the
eexpression of genes belonging to the cell cycle and apoptosis
category (see Supplemental Tables 1 and 2) may have been
influenced by the viral sequence insertion. However, most
genes present in the networks (vide below) are probably
minimally influenced by the presence of T.

Using the Ingenuity software, we found two major networks
with highly significant scores >50. The network of 35 down-
regulated genes contained 10 transcripts positively implicated
in osteoblastic differentiation, proliferation, or transformation.
These data suggest that the intersection set of AFT024 and
BMC9 is representative of bona fide undifferentiated cells, in
contrast to the set of 2018 and BFC012, which appears to
represent cells already committed to the osteogenic pathway
albeit unable to complete it by mineralization when placed in
an osteogenic condition.

The network of 35 upregulated genes belonged to two
categories, transcripts for cytoskeletal molecules and transcripts
implicated in the regulation of the transcriptional machine
(including chromatin remodeling). Cytoskeletal mole-
cules included structural components of actin-based cytoskel-
eton, chaperones for actins and tubulins, and proteins involved
in the signaling pathways leading to the assembly/disassembly
of microtubules and microfilaments. Some of the molecules
implicated in the transcriptional machinery have been detected
in protein complexes associated with cytoskeletal components,
such as the NuA4 histone acetylase complex (15) or the BAF53
complex (34).

Networks have not been emphasized in previous transcrip-
tomic studies on stem cells (4, 21, 27, 33, 35, 38, 40, 42, 43,
49). The networks described in the present study may serve as
a basis for future experiments. First, one may assess whether
the same networks are found in MSCs from primary cultures
(polyclonal and clonal cultures). Second, one may investigate
whether the hyperexpression of some of the network cytoskel-
etal components (either structural, such as moesin or cytoplas-
mic actin 1, or regulatory, such as RhoA) induces the hyper-
expression or downregulation of some of the molecules impli-
cated in the transcription machinery and in the differentiation
pathways. As a final point, it will be essential to verify whether
the hyperexpression of a cytoskeletal component belonging to
the gene network of upregulated molecules improves or ham-
pers the differentiation and/or stromal potentials of the trans-
duced cells. Genetic regulatory networks provide essential
information on the generation of the different cell types (26).
Future experiments should indicate whether the gene networks
previously described are genetic regulatory networks (i.e.,
include molecules involved in positive or negative regulatory
loops) that determine the maintenance in a nondifferentiated
state of this particular stem cell type.

In conclusion, this study describes gene networks for stromal
MSCs from the two major hematopoietic sites that should
help discriminate this cell type from other stem cells (ES,
neural, hematopoietic, etc.) and help define the stem cell state.

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discussion on different methodological aspects of this work.

GRANTS

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Table 1. Stemness gene candidates

<table>
<thead>
<tr>
<th>Trafficking</th>
<th>Cytoskeleton</th>
<th>Metabolism</th>
<th>Signaling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cops4</td>
<td>Actb</td>
<td>Acadin</td>
<td>Catnal</td>
</tr>
<tr>
<td>Kpna2</td>
<td>Ccl5</td>
<td>Fasn</td>
<td>Hdgf</td>
</tr>
<tr>
<td>Laptm5</td>
<td>Cct5</td>
<td>Nme2</td>
<td>Lybe</td>
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<td>Smc2</td>
<td>Tpm3</td>
<td>Scl25a5</td>
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<td>Syt11</td>
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<td></td>
<td>Ywhaq</td>
</tr>
</tbody>
</table>

Cell Cycle and Apoptosis | Protein Synthesis and Degradation | Transcription and Chromatin Remodeling

| Ccnb1 | Eif4g2 | Cpsf3 |
| Ccn5l | Hspa8  | Gata2 |
| Diabo | Pgf    | Hmn2  |
| Hipk3 | Psmb4  | Hrpap1|
| Nasp  | Psmc5  | Hnrpu |
| Ccnb1 | Sdf4   | Psip1 |

Transcripts expressed in stromal mesenchymal stem cells (MSCs) and in other stem cell types. For details, see Supplemental Table 3 (supplemented data are available at the online version of this article).
REFERENCES


