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## Distinct hematopoietic support by two human stromal cell lines

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**Objective.** The hematopoietic microenvironment is complex, and the role of myofibroblast in its function is crucial. In order to obtain a stable model reflecting this particular cell type, we have previously established human bone marrow cell lines from primary myofibroblastic Stro1<sup>+</sup> population (pStro1<sup>+</sup>). We placed HPV16 E6 and E7 expression under the control of different promoters. Here, we have characterized and studied the hematopoietic support for two cell lines corresponding to the promoters  $\alpha$ -SM ( $\alpha$ SM-56 line) and SV40 (SV40-56 line).

**Materials and Methods.** The expression profile was analyzed at the RNA level by gene array and at the protein level by Western blot, flow cytometry, and ELISA. Hematopoietic support determined using colony-forming unit (CFU) and stroma-adherent colony-forming cell (SA-CFC) assays.

**Results.** The phenotype of cell lines was not significantly modified compared with primary myofibroblastic cells. They secreted a broad spectrum of hematopoietic cytokines and nonspecific mediators. The two lines allowed the growth of hematopoietic precursors and had different support capabilities.

**Conclusions.** We have extensively characterized two novel human bone marrow stromal cell lines. They retained a myofibroblastic phenotype and have substantial but different hematopoietic support capabilities. These lines provided a basis for determining stromal factors involved in stem-cell regulation. © 2001 International Society for Experimental Hematology. Published by Elsevier Science Inc.

The study of the human microenvironment is essential for an understanding of the proliferation and differentiation of hematopoietic stem cells. In long-term bone marrow culture (LTBMC), these related processes depend on the development of an adherent stromal layer [1]. The fact that hematopoiesis in vivo is essentially confined to certain tissues stresses the need for a specific microenvironment [2].

The cellular complexity of the LTBMC system has led to attempts to culture selected populations. Subpopulations have been established by successive passaging of isolated stromal colonies [3], or by antibody selection of a specific cell lineage [4,5]. These cells unfortunately have a limited lifespan and their selection did not assure the purity of the population. Many groups have therefore turned to the use of immortalized stromal cell lines, most deriving from murine

tissues (for review see [6]). Some support human hematopoiesis but the addition of human cytokines is often required [7,8]. Moreover, species specificity is of paramount importance to studies on cell interaction.

The availability of human stromal cell lines would allow a stable and a well-defined tool to study the mechanisms of the support and eventually the manipulation of in vitro human hematopoiesis. To our knowledge, few human lines have been described. Those already established have been made using the SV40 virus or a part of it, and do not maintain hematopoiesis to any great extent [9–11]. When the expression of SV40 T antigen is controlled by an inducible promoter, cell lines allow long-term hematopoiesis, exhibiting a transformed phenotype only when the promoter is active, but this system is often leaky [12].

We have already demonstrated that in LTBMC, the major cell type was myofibroblastic [13]. In order to study the hematopoietic support by this cell type, we have established human stromal cell lines deriving from primary Stro1<sup>+</sup> (pStro1<sup>+</sup>) myofibroblastic cells (unpublished data). This model

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will be used to determine which molecules regulate hematopoiesis by characterizing cell lines with different hematopoietic capabilities. These cell lines were immortalized using the HPV16 E6 and E7 oncogenes, since a previous report has shown that human bone marrow stromal cell lines expressing these oncogenes maintain their capacity to support hematopoiesis [14]. The oncogene expression was placed under the control of the SV40 promoter (SV40-56 line), of the tissue-specific  $\alpha$ SM actin promoter ( $\alpha$ SM-56 line) or of the retroviral promoter long terminal repeat (LTR) (LTR-9 line). We have demonstrated that cells were less transformed when the  $\alpha$ SM actin physiological promoter is employed (unpublished data). Therefore such cells ( $\alpha$ SM-56 line) should be more representative of the normal human microenvironment.

Here, we reported the characterization and the functional properties of two stromal cell lines:  $\alpha$ SM-56 and SV40-56. The hematopoietic support of the LTR-9 line was not studied since it was not possible to control the growth with mitomycin C. Moreover, its phenotype indicated that this cell line is more transformed. The other cell lines retained most characteristics of myofibroblasts. They allowed the *in vitro* amplification of cord blood CD34<sup>+</sup> cells (nonadherent cells, SA-CFC) and resulting progenitors (CFU) but with distinct kinetics. In order to try to understand the observed differences between the two lines, we have also analyzed the expression of proteins potentially involved in the relationship between stromal and hematopoietic cells. These cell lines will therefore be good *ex vivo* models to provide information for stromal regulatory mechanisms of human hematopoiesis.

## Materials and methods

**Stromal cell culture Cell lines.** Human cell lines were established as previously described (unpublished data). Briefly, Stro1<sup>+</sup> selected cells were transduced with vectors containing HPV16 E6 and E7 oncogenes placed either under viral (SV40, LTR) or the human tissue-specific  $\alpha$ SM actin gene promoters. They were cultured in Dexter conditions using long-term culture medium: McCoy's medium supplemented with 12.5% heat-inactivated fetal calf serum (FCS, Seromed, Biochrom, Berlin), 12.5% heat-inactivated horse serum (HS, Life Technologies, Gaithersburg, MD, USA), 1% sodium bicarbonate, 1% sodium pyruvate, 0.4% MEM nonessential amino acids, 0.8% minimal essential medium (MEM) essential amino acids, 1% vitamin solution, 1% L-glutamine (200 mM), 1% penicillin-streptomycin solution (all solutions from Life Technologies), 10<sup>-6</sup> M hydrocortisone (Sigma Chemical Co., St. Louis, MO, USA), and 2.5 ng/mL of basic fibroblast growth factor (hr bFGF, R&D Systems, Abingdon, UK) at 37°C and 4% CO<sub>2</sub> in a humidified atmosphere.

**Primary culture.** Stro1<sup>+</sup> cells were isolated as previously described [5]. Briefly, after 10–14 days of culture, the adherent layer was treated with 3 mL of type I collagenase (250 U/mL, Sigma) for 20 minutes at 37°C. The supernatant was collected and washed three times in phosphate-buffered saline (PBS, Life Technologies) with 0.1% (w/v) of bovine serum albumin (BSA, Sigma). Coated

beads were then added to the cell suspension (3 beads per cell) and targeted cells were recovered with a magnetic particle concentrator (MPC-1, Dynal, Oslo, Norway). Cells were washed three times in 0.1% BSA/PBS, resuspended in long-term culture medium, and seeded at 0.5 × 10<sup>6</sup> cells per 25 cm<sup>2</sup> flask.

### Phenotype analysis

Cell line phenotype was studied after cells were propagated for more than 90 passages, and pStro1<sup>+</sup> cells were analyzed 14 days after isolation.

**Western blot.** Blots were performed as previously described [13]. Fully confluent adherent layers were detached in 10 mM EDTA-PBS and sonicated in the same buffer containing a protease inhibitor cocktail. Protein content was determined using the bicinchoninic acid protein assay reagent (BCA kit, Pierce Chemical Co., Rockford, IL, USA). The samples were mixed with an equal volume of 2× concentrated sample buffer, consisting of 4% (w/v) sodium dodecyl sulfate (SDS), 125 mM Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (v/v)  $\beta$ -mercaptoethanol, and 0.05% (w/v) bromophenol blue, and heated at 100°C for 5 minutes. Proteins were separated in a SDS-polyacrylamide gel (SDS-PAGE). The concentration of polyacrylamide was 10% for actins, calponin, and vimentin; 7.5% for desmin and cytokeratin 18 (CTK 18); 6% for caldesmons, vinculins, laminins, fibronectins, thrombospondin, and von Willebrand Factor (vWF); and with a reticulation rate reduced to 1.33% in a 6% gel slab for the heavy chains of myosin. Twenty  $\mu$ g of protein were loaded per lane. After SDS-PAGE, proteins were electrophoretically transferred onto a nitrocellulose membrane. Membranes were then incubated for one hour in 5% (w/v) nonfat dried milk in Tris-buffered-saline (TBS) with 0.1% Tween 20 (TTBS), washed twice in TTBS, and incubated overnight at 4°C with the appropriate dilution of primary antibody. After three washes in TTBS, the membrane was incubated for 1 hour with a horseradish peroxidase-linked secondary antibody or a biotinylated secondary antibody (Amersham, Little Chalfont, UK) and washed three times in TTBS. Blots were developed 1) directly with 4-chloro-1-naphthol for abundant proteins (actins, vimentin, fibronectins, laminins, vWF), or 2) after a 30-minute incubation with streptavidin-horseradish peroxidase for vinculins, desmin, thrombospondin-1 and CTK 18, or 3) using the chemiluminescent method (ECL kit, Amersham) for caldesmons, myosin heavy chains, and laminin  $\beta$ 1. For each protein, an internal reference control protein was used to minimize variation due to sample loading and staining. These internal controls were l-caldesmon for h-caldesmon, vinculin for metavinculin, nonmuscle myosin for smooth-muscle myosin SM1, and  $\beta$  cytoplasmic actin for the others. For quantifying the band intensity, blots or films were scanned with an Ultrascan laser densitometer (Pharmacia-LKB, Uppsala, Sweden) and analyzed with Gelscan XL software [15]. Antibodies and conditions used for Western blotting are shown in Table 1.

**Flow cytometry.** Cell suspensions of stromal cells (primary cells and lines) were washed twice in PBS, pelleted by centrifugation, and resuspended in 0.5% BSA/PBS. The cells were incubated with the appropriate primary antibody or the nonspecific isotype control for 30 minutes at 4°C. After two washes in PBS, cells were incubated with the secondary antibody for 30 minutes at 4°C. Antibodies used are shown in Table 2.

Cells were then analyzed using a FACSort flow cytometer (Becton-Dickinson, Mountain View, CA, USA); data acquisition and

**Table 1.** Antibodies used for Western blotting studies

Antigen	Abbreviation*	MW (kDa)	Antibodies	
			Clone	Source
β Actin	β	45	AC-15	Sigma <sup>†</sup>
Fibronectins	FNp	230	poly	Dako <sup>‡</sup>
EDa Fibronectin	EDaFN	230	3E2	Sigma
EDb Fibronectin	EDbFN	230	BC1	Dr. Charbord
Laminins	LMP	200, 220	/	IP <sup>  </sup>
Laminin β1	LMβ1	220	Rat	Chemicon <sup>§</sup>
Laminin β2	LMβ2	200	C4	IHB <sup>¶</sup>
Vimentin	Vim	55	13-2	Sigma
Thrombospondin-1	TSP	140	/	Dr. Charbord
αSM Actin	ASMA	45	1A4	Sigma
Vinculin	V	130	11-5	Sigma
metaVinculin	mV	150	11-5	Sigma
SM22	SM22	22	C1E11	Dr. Charbord
Calponin	Calp	34	hCP	Sigma
l Caldesmon	l Cald	75	C21	Sigma
h Caldesmon	h Cald	137	C21	Sigma
SM1 Myosin	SM1	204	h SMV	Sigma
NM Myosin	NMM	197	5A6	Dr. Charbord
Desmin	Des	55	DER11	Dako
Myosin light chain kinase	MLCK	138	K36	Sigma
Cytokeratin 18	CTK 18	45	CY90	Sigma
von Willebrand factor	vWF	220	/	Dako

Table inspired from [15] and completed with additional antibodies.

\*: used in Table 3; †: St. Louis, MO, USA; ‡: Dakopatts, Glostrup, Denmark; ||: IP, Pasteur Institute, Paris, France; §: Temecula, CA, USA; ¶: Iowa Hybridoma Bank, IA, USA.

analysis were performed with CellQuest software for 10,000 events. Positive events were counted for a fluorescent peak superior to that of the isotypic control. Only antigens with a mean fluorescence intensity (MFI) ratio (antigen/isotype) over 2 were considered positive.

**ELISA.** The supernatants were collected and stored at  $-30^{\circ}\text{C}$  until cytokine determination. The cytokine concentration of interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) was determined using ELISA kits purchased from Diaclone (Besançon, France), while granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), leukemia inhibitory factor (LIF), macrophage inflammatory protein-1α (MIP-1α), and Flt3-L concentration was assessed using kits purchased from R&D Systems following manufacturer's recommendations.

**Expression array.** The expression array was performed according to the manufacturer's recommendations (R&D Systems). Briefly, polyA<sup>+</sup> RNAs were isolated using oligo-dT coated beads (Dynabeads mRNA DIRECT kit, DYNAL). A reverse transcription was made with α-<sup>32</sup>P dCTP (Amersham Pharmacia Biotech, Saclay, France). An equal amount of radiolabeled cDNA probes was hybridized with each membrane. Membranes were analyzed using the multianalyst software (Biorad, Ivry-sur-Seine, France).

#### CD34<sup>+</sup> cell purification

Cells were obtained from normal human umbilical cord blood (HUCB) scheduled for discard after delivery of the infant. Mononuclear cells were isolated from HUCB using Ficoll-Hypaque

(Lymphoprep,  $1.077 \pm 0.001$  ng/mL; Nycomed, Oslo, Norway) density gradient purification. CD34<sup>+</sup> cell purification utilized positive selection using a CD34 progenitor isolation kit (QBEND/10) and a MACS immunomagnetic separation system (Milteny Biotec Inc, Paris, France) according to manufacturer's instructions.

#### HUCB CD34<sup>+</sup> cryopreservation and thawing

CD34<sup>+</sup> cells purified from HUCB were frozen in FCS supplemented with 10% dimethylsulfoxide (Sigma) using a step-down freezing procedure and placed in liquid nitrogen. Before use, samples were thawed, washed twice, and viability was determined using trypan blue. Only samples containing more than 98% viable cells were used in studies.

#### Cell sorting of CD34<sup>high/+</sup>CD38<sup>low/-</sup> populations

Cell sorting was performed with a FACSort flow cytometer (Becton-Dickinson). Thawed HUCB-CD34<sup>+</sup> cells were stained with phycoerythrin (PE)-conjugated CD34 (HPCA2, Becton-Dickinson) and fluorescein isothiocyanate (FITC)-conjugated CD38 (T16, Immunotech, France) monoclonal antibodies. The CD34<sup>high/+</sup>CD38<sup>low/-</sup> cell population was defined as to high CD34 antigen expression and CD38 expression less than the maximum FITC fluorescence of the control isotype. The CD34<sup>high/+</sup>CD38<sup>low/-</sup> population usually represented 3–5% of the isolated HUCB-CD34<sup>+</sup> cells. Sorting gates are shown in Figure 1.

#### Long-term support of colony-forming cells (CFC)

Stro1<sup>+</sup> cells and stromal cell lines were seeded in 12.5 cm<sup>2</sup> flasks coated with 0.5% gelatin (Sigma) in PBS and treated with mitomycin C (Sigma) when they reached 90% confluence. The mitomycin

**Table 2.** Antibodies used in phenotype analysis by cytometry

Antigen	Antibody name	Source
Stromal	Stro1	IHB
Fibroblast	1B10	Sigma
Stromal	CD10	Immunotech <sup>‡</sup>
Stromal	CD13	Immunotech
Mesenchymal cell antigen	SH3	Dr. Charbord
Mesenchymal cell antigen	SH2	Dr. Charbord
Fibroblast antigen	AS02	Dianova
HCA	F84	Dr. Charbord
CD44	A3D8	Sigma
VCAM-1	1G11	Immunotech
ICAM-1	6.5B5	Dako
ICAM-2	B-T1	Immunotech
$\alpha$ 1 integrin subunit	TS2/7	T Cell Science <sup>  </sup>
$\alpha$ 2 integrin subunit	gi9	Immunotech
$\alpha$ 3 integrin subunit	M-kid2	Immunotech
$\alpha$ 4 integrin subunit	HP2.1	Immunotech
$\alpha$ 5 integrin subunit	SAM-1	Immunotech
$\beta$ 1 integrin subunit	K20	Dako

\*: stock solution: 1 mg/mL in PBS with 1% (w/v) dimethyl sulfoxide; <sup>†</sup>: Southern Biotechnology Associates, Birmingham, AL, USA; <sup>‡</sup>: Immunotech: Marseille, France; <sup>||</sup>: Cambridge, MA, USA.

C concentration was 0.5  $\mu$ g/mL for primary Stro1<sup>+</sup> cells or the  $\alpha$ SM-56 line and 1  $\mu$ g/mL for the SV40-56 line. Cells were then washed twice with PBS before addition of fresh medium. Twenty-four hours later,  $1 \times 10^4$  CD34<sup>high/+</sup>CD38<sup>low/-</sup> sorted cells were seeded and demidepleted weekly for 5 weeks. CFC quantification was performed for the nonadherent cells harvested each week as described below.

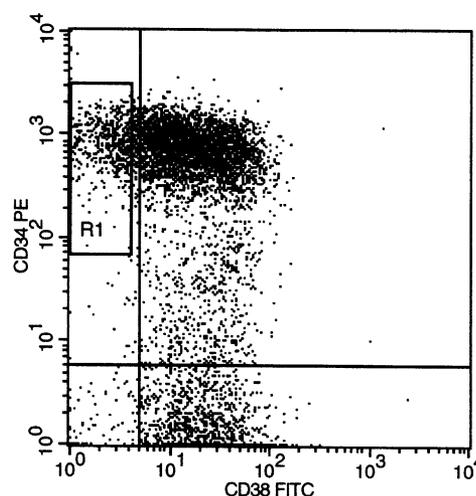
#### Colony assays

The cells were plated in methylcellulose (1.25% (w/v) final concentration in Iscove's medium, Sigma) supplemented with 2% FCS, 1.5% (w/v) deionized BSA, 300  $\mu$ g/mL transferin, 10  $\mu$ g/mL insulin,  $10^{-4}$  M  $\beta$ -mercaptoethanol, 28  $\mu$ g/mL calcium chloride, 15  $\mu$ g/mL each of cholesterol, oleic acid, and DL- $\alpha$  phosphatidyl cholin (all from Sigma), 5 ng/mL recombinant human IL-3 (rhIL-3), 5 ng/mL rhIL-6, 10 mg/mL rhG-CSF, 1 ng/mL rhGM-CSF, 50 ng/mL recombinant human stem-cell factor (rhSCF) (all cytokines from R&D Systems), and 1 U/mL recombinant human erythropoietin (rhEPO, Boehringer Mannheim, Meylan, France). One hundred to  $2 \times 10^4$  cells were plated, depending on the recovery and the culture time, at 37°C in humidified atmosphere. The colonies were scored after 14 days. Colonies of more than 40 nonhemoglobinized cells were scored as colony-forming unit-granulocyte macrophage (CFU-GM), while aggregates of more than 40 hemoglobinized cells were scored as burst-forming unit erythroid (BFU-E).

The yield of nucleated cells and progenitors was corrected weekly for demidepletion with the formula  $R_k = 2^k \times (N_k/N_0)$ , where  $R_k$  is the corrected yield for week  $k$  (as related to week 0),  $N_k$  is the number of cells collected at week  $k$ , and  $N_0$  is the number of cells seeded at day 0. Results are shown in Figure 2.

#### Stroma-adherent colony-forming cells

The ability to support stroma-adherent colony-forming cells (SA-CFC) was quantitatively evaluated as previously described [5,16]. Tamayo et al. [5] have demonstrated that their frequency was 1 for 167 CD34<sup>+</sup> cells and that one SA-CFC contains 5 to 40 CFU-GM.



**Figure 1.** Sort using flow cytometer for CD34<sup>high/+</sup>CD38<sup>low/-</sup> cells. Thawed CD34<sup>+</sup> cells were selected in function of their CD38 expression. Dot plot represents the relative fluorescence FL1 vs FL2. The R1 region corresponded to the sorted cells. A representative dot plot is shown.

Ninety percent confluent layers, cultured in 96-well plates, were treated with mitomycin C as described for 2.5 hours at 37°C. They were washed twice with PBS before addition of fresh medium. Twenty-four hours later, 20 wells were seeded with sorted CD34<sup>high/+</sup>CD38<sup>low/-</sup> cells at each of the following concentrations: 5, 15, 25, 50, 100, 200 cells per well.

Each week, half of the medium was removed and replaced by fresh medium. The wells containing stroma-adherent colony were counted from week 2 to week 5. The fraction of negative wells  $P(0)$  was calculated each week. The logarithm of  $P(0)$  was plotted vs the number of cells seeded. Linearity was confirmed by regression analysis. The number of stroma-adherent CFC was then calculated assuming that, on the average, one stroma-adherent CFC had been placed in wells for plates where  $P(0) = 0.37$  ( $\log 37 = 1.56$ ). Six experiments were made for each stroma. Results are expressed as the number of SA-CFC for 100 cells seeded as a function of the weeks in culture (Fig. 3).

## Results

### Phenotypic studies revealed that the immortalized lines retained stromal and myofibroblastic characteristics

The expression of several specific proteins of the vascular smooth muscle (VSM)-pathway was quantified by Western blotting for the three lines (SV40-56,  $\alpha$ SM-56, LTR-9) and primary cells. Results are shown in Table 3. The following markers were not detected in pStro1<sup>+</sup> cells or in lines: ED $\beta$  fibronectin, metavinculin, SM22, calponin, SM1, desmin, vWF. The  $\alpha$ SM actin protein was not detected by Western blot in the lines but was present in pStro1<sup>+</sup> cells. However, we determined by flow cytometry that this protein was still expressed in the lines and at a higher level in the  $\alpha$ SM-56 line (unpublished data). All lines expressed ED $\alpha$  fibronectin,  $\beta$ 1 and  $\beta$ 2 laminin, vimentin, h-caldesmon, myosin light

chain kinase (MLCK), and cytokeratin 18. The expression pattern was similar in pStro1<sup>+</sup>,  $\alpha$ SM-56, and SV40-56 cells. In contrast, LTR-9 cells have clearly different expression levels for fibronectins,  $\beta$ 1 and  $\beta$ 2 laminins, and h-caldesmon.

The LTR-9 line seems to have the more transformed phenotype. This is consistent with the fact that this line rapidly formed more colonies in agar than the  $\alpha$ SM-56 and the SV40-56 lines (unpublished data). Moreover, the LTR-9 line growth cannot be arrested by mitomycin C treatment (unpublished data, 1999). Uncontrolled growth makes it impossible to study hematopoietic support, so we concentrated our efforts on the other lines. In this line, the oncogene expression is controlled by the retroviral promoter LTR. This promoter is assumed to be strong, leading to a high expression of the E6 and E7 oncoproteins. Large amounts of oncoproteins are responsible for important disturbances in cellular biology.

The  $\alpha$ SM-56 and SV40-56 lines still expressed the major stromal markers: the Stro-1 antigen, the lysosomal IB10 antigen, the metalloprotease endopeptidase CD10, the metalloprotease aminopeptidase CD13, the mesenchymal SH2 and SH3 antigens, and a general fibroblastic antigen AS02. All these molecules were equally expressed by the two lines and the original pStro1<sup>+</sup> cells (Table 4).

#### *The cell lines preserved the adhesion characteristics of bone marrow stromal cells*

We have quantified by flow cytometry the expression of a set of molecules involved in the adhesion of hematopoietic cells to stroma or of stroma to the extracellular matrix. Results are shown in Table 4.

The hematopoietic cell adhesion molecule (HCA) was expressed more by the  $\alpha$ SM-56 and pStro1<sup>+</sup> cells (89 and 73%, respectively) than by the SV40-56 cells (27%). The CD44 molecule expression was comparable in both lines and pStro1<sup>+</sup> cells (70%–90%).

VCAM-1, ICAM-1, and ICAM-2 were expressed by the lines but not by pStro1<sup>+</sup> cells. The most striking difference was observed for ICAM-1: 94% (MFI ratio = 30) for the  $\alpha$ SM-56 line cells compared to 15% (MFI ratio = 3) for the SV40-56 line cells.

The  $\alpha$  integrin subunits (1 to 5) were expressed by lines and pStro1<sup>+</sup> cells. However, the  $\alpha$ 2 and  $\alpha$ 4 subunits were clearly overexpressed in the lines when compared to pStro1<sup>+</sup> cells. The  $\beta$ 1 integrin subunit expression was similar in pStro1<sup>+</sup> cells and the two lines.

#### *The cell lines present the same profile of cytokines secreted as the pStro1<sup>+</sup> cells*

Cytokines or growth factors known to be involved in the differentiation and commitment of hematopoietic cells were searched. The cytokines TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-10, Mip-1 $\alpha$ , and LIF were not detected. The secretion levels of IL-6, IL-8, and M-CSF were respectively more than 300, 1000, and 2000 pg/mL for the  $\alpha$ SM56 and SV40-56 lines and pStro1<sup>+</sup> cells. The G-CSF level was  $73 \pm 5$  pg/mL for  $\alpha$ SM56,  $87 \pm 4$  pg/mL for SV40-56, and  $61 \pm 6$  for pStro1<sup>+</sup>. The GM-CSF level was  $25 \pm 4$  pg/mL for  $\alpha$ SM56,  $40 \pm 3$  for SV40-56, and  $21 \pm 1$  for pStro1<sup>+</sup>.

#### *The cell lines have different mRNA profiles*

Table 5 shows molecules expressed in pStro1<sup>+</sup> cells and in cell lines. Among 375 cDNAs probed, 104 were expressed by one or more cellular type(s). The highest number was found for the Stro1<sup>+</sup> cells (69), vs 62 for the  $\alpha$ SM-56 line and 51 for the SV40-56 line. The genes can be classified in two categories. Firstly, there are those that have already been shown to be directly involved in hematopoiesis: macrophage-inhibitory cytokine-1 (MIC-1), M-CSF, tumor necrosis factor- $\beta$  (TNF- $\beta$ ), vascular endothelial growth factors (VEGF-A and -B), endoglin, growth-related oncogene protein (GRO- $\alpha$ , - $\beta$ , - $\gamma$ ), insulin-like growth factor (IGF), insulin-like growth factor binding protein (IGF-BP), gp130, and neuropilin-1. Secondly, there are the others that yet have no known role in hematopoiesis: midkine, osteoprotegerin, cadherin 11 (CAD11), decorin, the chemokine receptor CCR-5, the extracellular matrix metalloproteinase inducer (EMMPRIN), matrix metalloproteinases (MMP), and tissue inhibitors of metalloproteinases (TIMP).

The greatest difference between lines and pStro1<sup>+</sup> were for IGF-II, IGF-BP4, IGF-BP7, IGF-BP8, TIMP1, CAD11, decorin, and osteoprotegerin.

#### *The cell lines allow proliferation of primitive CD34<sup>high</sup>/<sup>+</sup>CD38<sup>low</sup>/<sup>-</sup> hematopoietic precursors*

To investigate the hematopoietic-supportive capacity of the cell lines, long-term coculture of sorted hematopoietic cells was carried out on confluent layers treated with mitomycin C. We evaluated the myelopoiesis in Dexter-type culture conditions. Results for four experiments are shown in Figure 2.

The number of nonadherent cells from coculture collected each week is represented in Figure 2A. The hemato-

**Table 3.** Western blotting results for cell lines and for pStro1<sup>+</sup> nontransformed cells

Line	FNp	FN EDa	LMNp	LMN $\beta$ 1	LMN $\beta$ 2	Vim	TSP	ASMA	h Cald	MLCK	CTK18
pStro1 <sup>+</sup>	1.06*	1.56	1.25	0.26	0.74	1.01	0.98	1.29	0.20	2.78	0.00
$\alpha$ SM-56	1.31	4.56	0.46	0.93	3.03	0.75	1.54	0	0.15	2.09	0.46
SV40-56	1.06	2.73	1.16	0.81	0.24	1.19	0.19	0	0.06	0.48	0.05
LTR-9	4.26	3.18	0.62	2.41	3.92	1.23	0	0	4.02	0.23	0.54

\*: expressed in arbitrary unit (u.a.) of densitometry report.

**Table 4.** Expression of stromal markers and adhesion molecules

Antigens	Cells		
	pStro1 <sup>+</sup>	αSM-56	SV40-56
Stro1	34.1 ± 10.2	62.0 ± 11.6	48.2 ± 4.4
1B10	45.0 ± 1.5	89.1 ± 3.9	49.3 ± 9.6
CD10	98.4 ± 0.5	98.5 ± 0.2	98.8 ± 0.1
CD13	99.3 ± 1.2	99.1 ± 0.0	99.0 ± 0.0
SH2	79.0 ± 5.2	66.8 ± 8.1	87.5 ± 0.9
SH3	92.0 ± 2.7	84.4 ± 2.6	90.2 ± 3.7
AS02	91.9 ± 0.9	97.4 ± 0.5	96.9 ± 1.6
HCA	73.2 ± 2.9	89.0 ± 4.7	26.8 ± 2.7
CD44	95.5 ± 0.9	97.3 ± 1.3	74.4 ± 23.9
VCAM-1	2.7 ± 0.4	22.3 ± 8.7	21.5 ± 1.6
ICAM-1	1.5 ± 0.3	94.0 ± 19.1	15.1 ± 9.0
ICAM-2	1.9 ± 0.6	13.7 ± 2.4	27.8 ± 3.2
α1 integrin subunit	80.0 ± 2.1	99.0 ± 0.0	92.5 ± 0.1
α2 integrin subunit	13.3 ± 1.2	98.1 ± 1.6	84.4 ± 2.7
α3 integrin subunit	59.8 ± 2.7	98.2 ± 1.6	91.9 ± 3.2
α4 integrin subunit	2.9 ± 0.4	82.2 ± 1.5	71.4 ± 3.2
α5 integrin subunit	65.0 ± 0.3	98.8 ± 0.0	92.5 ± 6.2
β1 integrin subunit	93.6 ± 2.3	99.0 ± 0.0	99.0 ± 0.1

Values are expressed as percentage of positive cells.

poietic cells did not survive in suspension in the same medium without the stromal support layer. Hematopoietic cells were produced for 5 weeks on the αSM-56 and SV40-56 lines, and for 6 weeks on pStro1<sup>+</sup>. The maximum proliferation was at 3 weeks for SV40-56 (270-fold increase compared to seeded cells), at week 4 for αSM-56 (420-fold increase), and at week 6 for pStro1<sup>+</sup> (870-fold increase).

We measured also the proportion of CFU-GM and BFU-E in these nonadherent fractions (Fig. 2B and C). CFU-GM were detected for 4 weeks for both lines and for 6 weeks for pStro1<sup>+</sup>. The maximum proliferation of progenitors occurred after 3 weeks on all cells with 22-, 17-, and 10-fold increases respectively for the SV40-56 line, pStro1<sup>+</sup> cells, and the αSM-56 line. The αSM-56 line and pStro1<sup>+</sup> cells had similar kinetics, though αSM-56 produced fewer CFU-GMs. The SV40-56 CFU-GM production pattern was clearly different, since this potential substantially decreased after 3 weeks.

The SV40-56 line and pStro1<sup>+</sup> cells had very similar potentials in inducing erythrocytic commitment, whereas the αSM-56 line did not support this lineage. The BFU-E number was increased by 3.1 at week 3 and 3.6 at week 2, on the SV40-56 line and pStro1<sup>+</sup> cells respectively.

#### *Different patterns of SA-CFC production were observed between support layers*

The kinetics of colony formation are shown in Figure 3.

In order to determine the capacity of stromal cells to sustain hematopoietic colony formation, hematopoietic cells were seeded in limiting dilutions on stromal layers (pStro1<sup>+</sup> cells or cell lines).

The number of colonies generated per 100 hematopoietic cells seeded was calculated each week for 6 weeks. SA-CFC

**Table 5.** Molecules expressed at the mRNA level

Molecules	Cells		
	Stro1 <sup>+</sup>	αSM-56	SV40-56
MIC-1	4.3	9.2	36.4
M-CSF	6.8	6.8	10.0
TNF-β	6.9	5.1	4.8
VEGF-A	12.2	54.9	16.3
VEGF-B	15.3	22.8	13.8
Endoglin	37.5	13.6	7.2
GRO α	0	4.2	0
GRO β	0	3.4	0.8
GRO γ	0	10.5	5.4
IGF-II	166.5	0	0
IGF-BP4	359.8	19.4	51.7
IGF-BP6	0	27.4	0
IGF-BP7	136.8	5.4	4.6
IGF-BP8	119.9	0	12.4
gp130	11.1	0	9.7
Neuropilin-1	11.3	6.1	10.5
Midkine	6.0	38.8	0
Osteoprotegerin	13.8	67.0	17.2
CAD11	32.1	0	0
Decorin	118.7	9.8	5.7
CCR-5	0	0	10.1
EMMPRIN	24.0	12.3	0
MMP1	0	0	26.5
MMP14	10.2	0	10.5
TIMP1	341.0	50.2	43.9
TIMP3	26.0	7.3	31.2

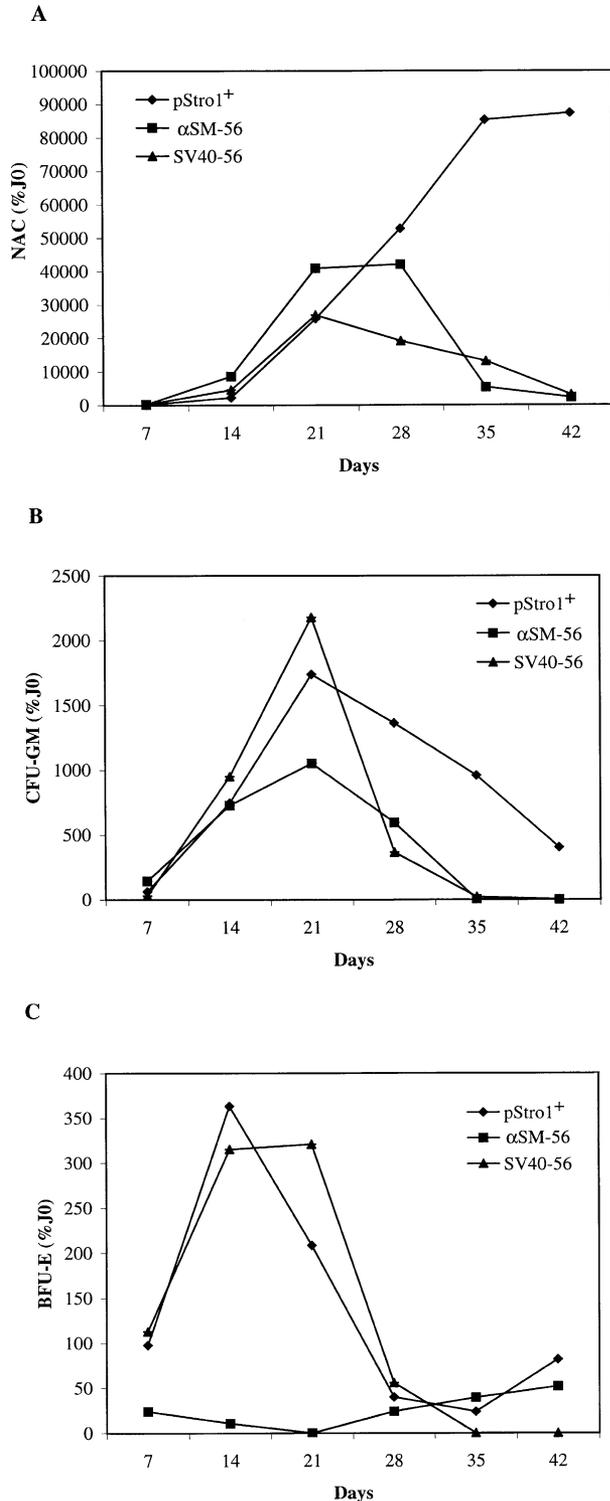
The results are expressed in arbitrary units (u.a.), as the relative value compared to housekeeping genes (GAPDH, β 2 microglobulin, β actin, cyclophilin A, HLA A, α tubulin).

were observed up to 6 weeks in all cases. The colony formation patterns of αSM-56 line and pStro1<sup>+</sup> cells were very similar, being maximal at week 3 (9 and 6 colonies per 100 seeded cells, respectively). The SV40-56 line peaked for colony formation at weeks 3 and 4 (5 colonies per 100 seeded cells).

## **Discussion**

The aim of this work was to identify factors involved in the control of proliferation and differentiation of hematopoietic cells. We have established two human myofibroblastic stromal cell lines using the HPV 16 E6 and E7 oncoproteins, αSM-56 and SV40-56. Two different promoters were used in these cell lines for controlling the oncogene expression, originating from the αSM actin gene and the SV40 virus. Here, we have characterized and studied the hematopoietic support for these two lines compared to pStro1<sup>+</sup> in order to correlate phenotype with functions.

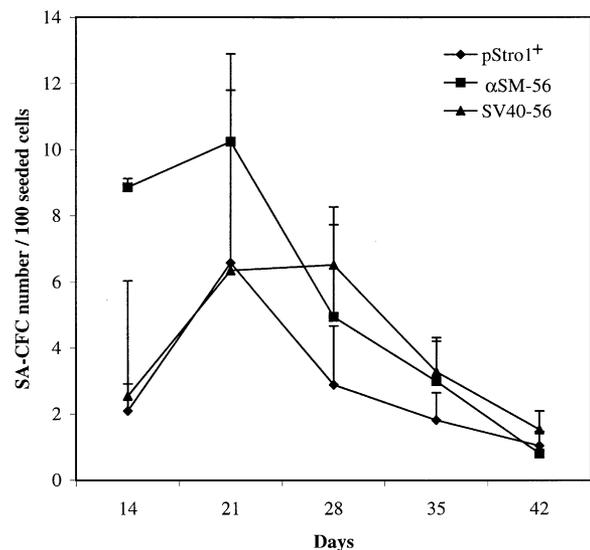
Primary bone marrow stromal cells follow a VSM differentiation pathway marked by the expression of fibronectin, laminin, vimentin, thrombospondin, αSM actin, h-caldesmon, and MLCK [13,17–20]. Our cell lines, deriving from pStro1<sup>+</sup> cells, displayed these markers. The lines, but not the pStro1<sup>+</sup> cells, expressed cytokeratin 18, a fetal marker



**Figure 2.** Cell lines were able to sustain hematopoiesis as nonimmortalized pStro1<sup>+</sup> cells. Results are expressed in % of day 0. (A) shows the percentage of nonadherent cells (NAC) recovered each week, (B) represents the percentage of CFU-GM, and (C) shows the percentage of BFU-E. Values represent the average  $\pm$  SEM of four experiments. [FCG]

of smooth-muscle cells [19]. The reexpression of oncofetal proteins has been well documented in transformed cells [21]. Although these lines were of VSM origin, some cells within the culture acquired adipocytic features as demonstrated by lipid accumulation (unpublished data, 2000). This showed that they have kept the capacity to differentiate into another stromal lineage. Such a capacity has also been previously described for primary Stro1<sup>+</sup> precursors [4].

Since physical stroma-hematopoietic progenitor cell interactions are essential in hematopoiesis [22], we also analyzed the expression of some adhesion molecules by the lines and by the pStro1<sup>+</sup>. The expression levels were identical for CD10, CD13, SH2, SH3, AS02,  $\alpha$ 1,  $\alpha$ 3,  $\alpha$ 5, and  $\beta$ 1 integrin subunits and different for VCAM-1, ICAM-1, ICAM-2,  $\alpha$ 2, and  $\alpha$ 4 integrin subunits, and HCA. Only ICAM-1 and HCA were differentially expressed between the two lines. VCAM-1 and ICAM-1 are inducible molecules not normally expressed at a significant level by pStro1<sup>+</sup> in resting conditions. Since VCAM-1 and ICAM-1 interact with integrin complexes on the hematopoietic cells [23–25], they could reinforce adhesion and therefore the hematopoietic support of the lines. Expression of HCA molecule was higher in the  $\alpha$ SM-56 line and pStro1<sup>+</sup> cells. HCA has been shown to be expressed both by hematopoietic cells [26] and by stromal cells in blood-forming tissues and promotes the adhesion of the most primitive hematopoietic cells [27]. The production of  $\alpha$ 2 and  $\alpha$ 4 integrin subunits was also greater by the lines than by the pStro1<sup>+</sup> cells. These molecules, which are associated with  $\beta$ 1 subunit, are responsible for the adhesion of stromal cells to collagen,



**Figure 3.** Different kinetics of SA-CFC appearance were observed between the lines. Sorted hematopoietic cells were cocultured with the lines in limiting dilution assay. Hematopoietic colonies were counted each week. The number of SA-CFC per 100 CD34<sup>high</sup>CD38<sup>low/-</sup> seeded is shown in function of the culture time. Values represented the mean  $\pm$  SEM of four experiments.

laminin, and fibronectin, and participate in the extracellular matrix organization [28]. Since the hematopoietic precursors adhere to the extracellular matrix, the capacity of stromal cells to produce and organize the extracellular matrix components is an essential component of hematopoiesis [29].

We have also examined the expression of different cytokines and growth factors. Some, such as TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-10, Mip1 $\alpha$ , and LIF, were not detected. However, the cell lines, similar to pStro1<sup>+</sup>, expressed cytokines normally associated with the maintenance of early or late hematopoiesis: IL-6, IL-8, M-CSF, G-CSF, GM-CSF, TNF- $\beta$ . VEGF-A and -B were also present. This cytokine expression pattern is similar to that of both nonimmortalized stromal cells [30] and other rare human bone marrow stromal cell lines [31,32,14].

Concerning the cytokine array, only the most relevant results will be discussed. Neuropilin-1, a VEGF pathway-associated molecule, was expressed in lines and pStro1<sup>+</sup> cells. The expression of VEGF by stromal cells (our data) could induce the expression of thrombopoietin (TPO) and Flt3-L within the lines. This induction has already been observed in the murine MS-5 line usually considered as the benchmark for hematopoietic support [33]. VEGF may also stimulate hematopoietic precursors that express the KDR receptor [34].

Endoglin, a component of the TGF- $\beta$  receptor system, was also found; it is usually considered as an endothelial marker but may also play a role in the interaction between stromal cells and hematopoietic cells during development [35] and could mediate the production of cytokine by stromal cells in response to TGF- $\beta$  [36,37], which in turn may also influence the VSM pathway [38].

IGF-II and the IGF-BP expression was higher in pStro1<sup>+</sup> cells. These molecules are produced by murine stroma [39] and are known to promote hematopoiesis [40].

The stromal cell lines  $\alpha$ SM-56 and SV40-56 allowed the proliferation of primitive hematopoietic cells from HUCB. The maximum release of nonadherent cells was at week 3 for the SV40-56 line, week 4 for the  $\alpha$ SM-56 line, and week 6 for the pStro1<sup>+</sup> layers. Thus the profiles of nonadherent cell production were different, being earlier for the two lines than the normal stromal layer. Moreover, the  $\alpha$ SM-56 line produced two times more cells than the SV40-56 at any time. As expected, the nonadherent cells were myeloid hematopoietic cells expressing CD45 and CD15 antigens but not CD3, CD19, or glycophorin-A (unpublished data, 2000).

The CFU-GM content within these expanded hematopoietic cells was increased 10-fold to 22-fold over the level at day 0. Proliferation was maintained for at least 4 weeks. The maximum was at week 3 for all layers, and it was two times greater from the SV40-56 line than from the  $\alpha$ SM-56 line. The SV40-56 line was responsible for the lowest release of nonadherent cells but produced more CFU-GM than the  $\alpha$ SM-56 line. Thus, the SV40-56 line maintained the precursors whereas the  $\alpha$ SM-56 line induced the matu-

ration and proliferation of hematopoietic cells. The major differences in hematopoiesis support for the lines concerned the erythrocytic commitment. The SV40-56 line had the same capacity as the pStro1<sup>+</sup> cells; in contrast, the  $\alpha$ SM-56 did not maintain this erythrocytic potential. This BFU-E support also confirmed the capacity of the SV40-56 line to maintain earlier precursors. The  $\alpha$ SM-56 and SV40-56 lines' capability to support hematopoiesis is greater than that of previously reported human lines [14] or indeed the murine MS-5 line, which produced a maximum progenitor increase of 10-fold to 12-fold of CFU-GM during the culture period [41,42].

The lines also differed in their capacities to support the development of stroma-adherent hematopoietic colonies. The kinetics of colony emergence were similar for the  $\alpha$ SM-56 and pStro1<sup>+</sup> cells, the number of colonies being maximal at 3 weeks. In contrast, for the SV40-56 line, the colony number was maintained at a maximum level for 2 weeks (weeks 3 and 4). These data suggested that the  $\alpha$ SM-56 line expressed inducers that stimulate progenitors into entry of the cell cycle, whereas the SV40-56 reduced the hematopoietic proliferation. This experiment confirmed the results obtained with the nonadherent cells, CFU-GM and BFU-E.

The major difference between pStro1<sup>+</sup> cells and the lines concerned the IGF system. Only the pStro1<sup>+</sup> cells secreted large amounts of IGF-II. IGF-II secretion may account for the higher nonadherent cell production in pStro1<sup>+</sup> cocultures than in lines coculture, since it augmented the proliferation of hematopoietic cells [39]. IGF-II, as IGF-I, is also known to be involved in the enhancement in erythroid colony formation [43]. The lack of IGF-II production by the  $\alpha$ SM-56 line could be in part responsible for the lack of erythrocytic commitment. The SV40-56 line has the capacity to support erythrocytic commitment, but interestingly this line does not produce IGF-II. We can therefore hypothesize that it may utilize an alternative pathway to IGF-II.

We have demonstrated here that we have established two human lines that support in vitro human hematopoiesis. Our experiments have shown that this support is different, in terms of proliferation or quiescence of progenitors. Roecklein et al. [14] have established several human stromal cell lines with distinct capacities. Our results confirm these observations and also indicate that this functional heterogeneity is retrieved in cell lines derived from Stro1<sup>+</sup> precursors. The medullary microenvironment heterogeneity could therefore originate from the differentiation of Stro1<sup>+</sup> precursors.

We have observed few phenotypic differences between the  $\alpha$ SM-56 and SV40-56 lines, which may explain the mechanism of hematopoietic support. Although these lines express a set of proteins and cytokines involved in hematopoiesis, it is difficult so far to assess the function of each molecule. Other molecules detected with the expression array, but without any clear implication in hematopoiesis, could also be involved in stem-cell biology. Further studies would focus on specific factors acting in controlling quies-

cence vs proliferation of hematopoietic precursors and other lines described for their capacity to sustain primitive precursor should be submitted to this cytokine array approach [44].

In addition, these lines also provide a starting point for the manipulation of human hematopoiesis. Indeed, there are many possibilities. For example, infection of SV40-56 line with a retrovirus expressing the human erythropoietin gene may produce a line capable of manufacturing fully differentiated erythrocytes from CD34<sup>+</sup> progenitors.

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