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Original Article

Long-Term Maintenance of Hematopoietic Stem Cells Does Not Require Contact with Embryo-Derived Stromal Cells in Cocultures

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ABSTRACT

We recently established that two midgestation-derived stromal clones—UG26-1B6, urogenital ridge–derived, and EL08-1D2, embryonic liver–derived—support the maintenance of murine adult bone marrow and human cord blood hematopoietic repopulating stem cells (HSCs). In this study, we investigate whether direct HSC-stroma contact is required for this stem cell maintenance. Adult bone marrow ckit$^+$ Ly-6C$^-$ side population (K6-SP) cells and stromal cells were cocultured under contact or noncontact conditions. These experiments showed that HSCs were maintained for at least 4 weeks in culture and that direct contact between HSCs and stromal cells was not required. To find out which factors might be involved in HSC maintenance, we compared the gene expression profile of EL08-1D2 and UG26-1B6 with four HSC-nonsupportive clones. We found that EL08-1D2 and UG26-1B6 both expressed 21 genes at a higher level, including the putative secreted factors fibroblast growth factor-7, insulin-like growth factor-binding proteins 3 and 4, pleiotrophin, pentaxin-related, and thrombospondin 2, whereas 11 genes, including *GPX-3* and *HSP27*, were expressed at a lower level. In summary, we show for the first time long-term maintenance of adult bone marrow HSCs in stroma noncontact cultures and identify some secreted molecules that may be involved in this support. STEM CELLS *2005;23:842–851*

INTRODUCTION

The first definitive hematopoietic repopulating stem cells (HSCs) are autonomously generated within the embryonic aorta, gonads, and mesonephros (AGM) region [1, 2]. In the midgestation mouse embryo, these HSCs are found mainly in the major blood vessels, the dorsal aorta, and the arteries [3]. Interestingly, HSCs expand dramatically between embryonic day 10.5 (E10.5) and E11 in the dorsal aorta and subsequently can be found in the urogenital ridges [3], yolk sac, and liver. Thereafter, the fetal liver shows a capacity to expand but not to induce additional HSCs [4]. Thus, the AGM microenvironment plays a unique role in the establishment of definitive hematopoiesis.

To delineate the molecular events involved in the induction and early events of definitive hematopoiesis within the AGM microenvironment, we and others have generated stromal cell lines from this region [5–8]. These lines were shown to support cultures of murine fetal liver–derived [5], marrow-derived [6, 8], and human cord blood–derived cells [6, 9]. More important, these lines maintained repopulating stem cells from these sources. Because for most of the lines nonsupportive counterparts were generated in parallel, these cell lines enable the comparison of gene expression patterns required for the maintenance of murine HSCs.

In a first comparison, most investigators focused on the expression of certain growth factors, which are thought to be of

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importance for the maintenance of hematopoiesis in culture. It was demonstrated that hematopoiesis-supportive AGM-derived stromal cells express mRNA for many growth factors [5–7]. However, factors that could distinguish hematopoiesis-supportive stromal cells from nonsupportive cells were not identified. From our own studies [7], it appeared that supportive cell lines expressed the chordin-like gene, the product of which is involved in the regulation of the activity of bone morphogenic protein 4 [10]. In addition, although a specific support-promoting cytokine could not be identified, hematopoiesis-supportive stromal cells more frequently expressed high mRNA levels of thrombopoietin (TPO), stem cell factor (SCF), and interleukin (IL)–6 [7]. Differences in cytokine expression between supportive stromal cells are also apparent. For instance, the AGM-S3 line expresses high levels of oncostatin M (OSM) but no M-CSF [6], whereas the endothelial DAS104-4 line expresses M-CSF [5]. The supportive lines we have generated (i.e., EL08-1D2 and UG26-1B6) fail to express OSM, but all express G-CSF [7], which is not found on AGM-S3 or DAS104-4. This divergence in growth factor transcript levels or gene expression patterns suggests that soluble factors like M-CSF, G-CSF, and OSM do not suffice for the maintenance of stem cells in culture.

For the development of clinically applicable regimens for the maintenance or expansion of stem cells, many investigators use soluble factors. Commonly used factors are TPO, Flk2-ligand (FL), SCF, IL-6, G-CSF, and IL-3. Although some regimens have met with a degree of success, most investigators reported only a moderate expansion of stem cells in culture. Indeed, many reports stress the fact that it is difficult, if not impossible, to maintain stem cells in culture without the presence of a supportive feeder layer of cells. Whether the stem cells should be in contact with these stromal cells is still a matter of debate. Both the AGM-S3 and DAS104-4 cell lines were incapable of maintaining early progenitors from fetal liver [5] or cord blood $CD34^+$ cells [6] when they were not in direct contact with the stromal cells during culture. Previously, we did not investigate the ability of the stromal cells we have generated to maintain bone marrow HSCs in noncontact cocultures. In this study, we demonstrate that the urogenital ridge–derived UG26-1B6 and the embryonic liver–derived cell line EL08-1D2 both support the maintenance of adult bone marrow stem cells in noncontact cultures. Gene expression analyses of two hematopoiesis-supportive (UG26-1B6 and EL08-1D2) and four nonsupportive cell lines (UG15-1B7, AM20-1B4, EL28-1B3, and AM30-3F4) have revealed several putative secreted molecules that might be involved in the maintenance of stem cells in noncontact culture.

Materials and Methods

Animals

(C57BL/10 X CBA)F1 mice (B10CBA, 8–16 weeks old) were used as recipients for transplantation experiments with Ln72 transgenic mice and for maintaining the transgenic mouse lines. Ln72 mice transgenic for the human β-globin locus [11] were used as donor cells in transplantation experiments. Mice were bred in the Experimental Animal Facility of the Erasmus University.

Stromal Cells and Cell Lines

Stromal cell lines from E10 and E11 embryos were cultured as described in detail previously [8].

Enrichment of Long-Term Hematopoiesis-Repopulating Cells

Bone marrow cells were labeled with Hoechst 33342 (Molecular Probes, Leiden, the Netherlands, http://probes.invitrogen.com) as reported [12]. After labeling with Hoechst 33342, cells were kept on ice and further labeled with a fluorescein isothiocyanate-conjugated antibody to Ly-$6C^-$ (ER-MP20) and phycoerythrin-conjugated anti-c-kit antibody (2B8, from BD-Pharmingen, Heidelberg, Germany, http://www.bdbiosciences.com/pharmingen) for 30 minutes. This was followed by two washes with cold H-F/2^+ (Hanks' balanced salt solution [Gibco, Karlsruhe, Germany, http://www.invitrogen.com], 2% fetal calf serum, and 10 mM Hepes [Gibco]). $Ckit^+$ Ly-6C-SP (K6-SP) cells (Fig. 1) were sorted on a FACSVantage SE (BD-Immunocytometry Systems, http://www.bdbiosciences.com/immunocytometry-systems). After sorting, viable cells were counted after a trypan blue stain (Sigma, Munich, Germany, http://sigmaaldrich.com) using a Neubauer hemocytometer (Paul Marienfeld, Bad Mergentheim, Germany, http://www.superior.de).

Contact and Noncontact Long-Term Cultures of HSCs

Cultures of stromal cell lines were grown to confluence in 0.1% gelatin-coated 35-mm dishes (Corning-Costar, Schiphol-Rijk, the Netherlands, http://www.corning.com/lifesciences) and irradiated at 30 Gy. For noncompetititive repopulation assays, 300 K6-SP cells (representing 30 repopulating cell equivalents) were seeded in 3-ml long-term culture (LTC) medium (M5300 [StemCell Technologies, Vancouver, British Columbia, Canada, http://www.stemcell.com], supplemented with Glutamax I [Gibco], hydrocortisone [10^{-6} M], and antibiotics) and cultured for 4–6 weeks. Each week one half of the medium was removed and replaced with fresh medium and hydrocortisone. At the end of the culture, the adherent and nonadherent cells were mixed and assayed for the presence of HSCs.

Noncontact cultures were set up in a slightly different manner. On top of the irradiated stromal cells, a membrane was placed (0.4-μm Transwell-COLL, Costar 3491) in which 300 K6-SP cells were seeded. Each week, one half of the medium was removed from beneath the membrane and replaced on top of the membrane with fresh medium and hydrocortisone. At the end of culture, whole cultures were harvested as described above and the adherent (underneath membrane) and nonadherent cells (top of membrane) were mixed and assayed for repopulating ability.

Assay for Long-Term Repopulating Ability

Repopulating ability of sorted and cultured Ln72 K6-SP cells was performed in lethally irradiated adult B10CBA recipients as previously described [1–3, 8]. At 6 and 16 weeks after transplantation, genomic DNA was isolated from peripheral blood samples. Genomic DNA was isolated and polymerase chain reaction (PCR) analysis was performed with oligos specific for the human β-globin transgene as previously described [8]. A recipient was considered positive only if more than 10% of the DNA content was of donor genotype. Estimates of the repopulating cell frequencies were obtained from the numbers of negative mice in each of the various cell dose groups using the method of maximum likelihood available in the L-Calc software (StemCell Technologies).

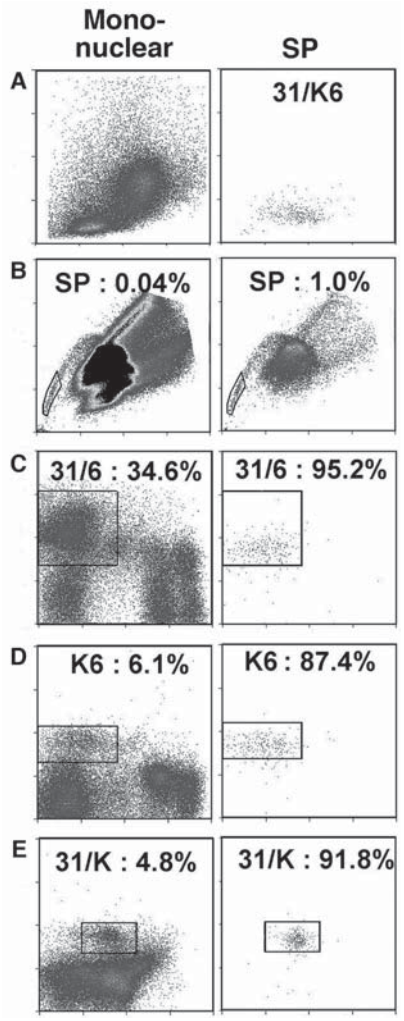

Figure 1. Sorting strategy for murine hematopoietic stem cells. Murine adult bone marrow (shown in **A** as dot plot of forward and side scatter) was stained with Hoechst 33342 to visualize the side population (SP) cells **(B)**. The left panel in **(B)** shows the SP in the K6 gate (see **D**). Subsequently, the SP cells were stained with antibodies against ckit (phycoerythrin conjugate), Ly-6C (fluorescein isothiocyanate conjugate), and CD31-TriColor. For sorting, the SP cell were depicted within the population of ckit$^+$ Ly-6C$^-$ cells (K6-SP). This population of cells (of which more than 90% also expressed CD31) is highly enriched in SP cells. Shown are representative dot plots of mononuclear cells (left panels) and SP cells: **(C)** CD31 (y-axis) and Ly-6C (x-axis); **(D)** ckit (y-axis) and Ly-6C (x-axis), and **(E)** ckit (y-axis) and CD31 (x-axis).

Preparation of Total RNA and Macroarray Analysis

Stromal cells were grown to confluency in stromal medium. The cells were then irradiated at 30 Gy on a Mevatron (Siemens, Munich, Germany, http://www.medical.siemens.com). The stroma medium was replaced completely with LTC medium. The irradiated cells were cultured for another week, after which the LTC medium was replaced completely with fresh medium. One day after the second medium replacement, cells were lysed in TRIzol (Invitrogen, Karlsruhe, Germany, http://www.invitrogen.com) and stored at −80°C until preparation of total cellular RNA. Total genomic DNA-free RNA was prepared as described by the manufacturer (Invitrogen). [^{32}P]-labeled cDNA was prepared and hybridized to Clontech Atlas Mouse 1.2 and Mouse 1.2 II cDNA expression arrays as described by the manufacturer (BD Biosciences Clontech, Heidelberg, Germany, http://www.clontech.com/clontech). Signal intensities of hybridized cDNA were measured 4 days to 1 week later using a phosphoimager (Molecular Dynamics, Amersham-Biosciences, Freiburg, Germany). Total RNA from two different passages of each of the six lines (EL08-1D2, UG26-1B6, UG15-1B7, AM20-1B4, EL28-1B3, and AM30-3F4) was hybridized. In this way, four biological replicates of hematopoiesis-supportive lines (two different passages of UG26-1B6 and EL08-1D2; samples 1 through 4) and eight biological replicates of nonsupportive lines (two different passages of UG15-1B7, AM20-1B4, EL28-1B3, and AM30-3F4; samples 5 through 12) were investigated. Altogether, 12 hybridizations were performed per array. Because two different arrays were used (Clontech Atlas M1.2 and M1.2 II), a total of 24 hybridizations were performed. Image analysis was performed using the AtlasImage 2.01 software Package (BD Biosciences Clontech). Background-subtracted mean intensities were normalized according to Huber et al. [13]. Signal intensities of the two array types (Atlas M1.2 and M1.2 II) were normalized separately, and normalized values of both filters were further analyzed together. To construct tables of differentially expressed genes, a two-sided *t*-test for each gene comparing samples 1 through 4 and 5 through 12 was performed. Genes with *p* values below .05 and estimated fold change above 2.0 were regarded relevant. Estimated fold changes are the mean of normalized signal intensities of samples 1 through 4 divided by the mean of normalized signal intensities of samples 5 through 12.

Reverse Transcription–PCR and Real-Time PCR Analysis

Total DNA-free RNA was prepared as described above. cDNA was prepared using Superscript RNAse H reverse transcription (Invitrogen) and poly-T primers (Invitrogen). For reverse tran-

scription (RT)-PCR, the equivalent of 0.5 μg RNA was amplified using Taq polymerase (Invitrogen) and the primers described in Table 1. Products were amplified for 35 cycles at 92°C (45 seconds), 60°C (1 minute), and 72°C (2 minutes), and products were visualized on a 1.5% agarose gel with ethidium bromide. For real-time PCR, the equivalent of 0.1 μg RNA was amplified using SYBR-Green PCR master-mix (Applied Biosystems, Darmstadt, Germany, http://appliedbiosystems.com) on an Abi Prism 7,700 Sequence Detection System (Applied Biosystems). The primers used in this study are shown in Table 1.

Insulin-Like Growth Factor–Binding Protein-3 ELISA

Embryonic stromal cells were irradiated and grown in the same way as for the array analysis. One to 6 days after the second medium change, the conditioned media were collected and stored at –20°C until use. The presence of insulin-like growth factor-binding protein (IGFBP)-3 in conditioned media was quantitated using a murine-specific IGFBP-3 ELISA (DuoSet [R&D Systems, Minneapolis, http://www.rndsystems.com]), as described by the manufacturer.

Results

We have shown that HSCs from the CD31$^+$ c-kit$^+$ Ly-6C$^-$ (CD31$^+$-K6) marrow fraction are maintained on the urogenital ridge–derived stromal clone UG26-1B6 [8]. In the present study, we wished to use an even more HSC-enriched population to minimize the effects of proliferating and metabolically active mature hematopoietic cells on HSC maintenance. For this purpose, we combined the CD31$^+$-K6 stain with Hoechst 33342 staining to obtain the side population (SP) cells, which represent approximately 0.04% of adult bone marrow cells (Fig. 1B). The c-kit$^+$ Ly-6C$^-$ bone marrow cells (Fig. 1C) (95% of these cells expressed CD31) are 20- to 50-fold enriched in SP cells (K6-SP cells, Fig. 1B). For the experiments described here, we used these K6-SP cells from adult bone marrow. The human β-globin transgene served as the donor cell marker to detect repopulation after in vivo injection into irradiated adult recipients [8]. As determined by in vivo transplantation, the frequency of HSCs in the K6-SP population was 1 in 10 (95% confidence interval, 1 in 14 to 1 in 7; Table 2) 16 weeks after transplant.

Previously, it was shown that the support of hematopoiesis in cocultures with two AGM-derived cell lines, AGM-S3 and DAS 104-4, depended on direct contact of stromal cells with the hematopoietic cells [5, 6]. However, UG26-1B6 and the cell line that supports maintenance of E11 HSCs [8] and human cobblestone area-forming cells [9], EL08-1D2, were not previously tested in noncontact cultures for support of colony-forming cells or HSCs. Thus, we cultured Lin- or K6-SP cells in collagen-coated transwell inserts above irradiated EL08-1D2 or UG26-1B6 for 4–6 weeks.

Table 2. Frequency of repopulating cells in murine bone marrow K6-SP cells

Number of cells per mouse	Positive/total	Percentage
3	7/13	54
10	20/31	64
30	12/14	86
100	5/5	100
HSC frequency	1 in 10	
95% CI, upper	1 in 7	
95% CI, lower	1 in 14	

Lethally irradiated B10CBA mice were injected in the tail vein with the number of male line 72 K6-SP cells indicated. In addition, each recipient was coinjected with 2×10^5 female recipient-type B10CBA spleen cells. After 16 weeks, peripheral blood was investigated for the presence of the transgenic human β-globin marker by semiquantitative polymerase chain reaction. Recipient mice with more than 10% donor marker in genomic DNA were designated positive.
Abbreviations: CI, confidence interval; HSC, hematopoietic repopulating stem cell; SP, side population.

Table 1. Primer pairs used in the RT-PCR analysis shown in this study

Gene	Sense primer	Antisense primer	Product size (bp)
Cathepsin K	GGG CCA GGA TGA AAG TTG	TGG CCA TGT TGG TAA TGC	362
sFRP-2	CTC TGC GTG CAG GTG AAG GA	AAA CGG TCG CAC TCC AGC AT	85
HSP27	CGG TGC TTC ACC CGG AAA TA	CGC TGA CTG CGT GAC TGC TT	135
IGFBP-3	CCA GCA CAC ACC GAG TGA CC	CGT CTT TCC CCT TGG TGT CG	380
IGFBP-4	ACA GGG CCC ATC TTT GGT CA	CTG TCC TTC CGG CCT TCC TC	106
Pentaxin-like (*PTX3*)	GTT GGT GGT GGG TGG AAA GG	TCC CCG GAT GTG ACA GGA TT	346
Pleiotrophin (*PTN*) #1	GCT GCC TTC CTG GCA TTG AT	TTG CCA CAG GGC TTG GAG AT	380
Pleiotrophin (*PTN*) #2	ACA CTC CAC TGC CAT TCT CC	TTT TCA TCT TGG CAG CTG TG	100
Thrombospondin 2 #1	ATC CCA AGG GGA CCA CAC AA	CAG GGC ATT CCT CAG GTG CT	344
GAPDH	CTT CAC CAC CAT GGA GAA GG	CCA CCC TGT TGC TGT AGC C	650
RPL-p0	TGC ATC CTG CAC CAC CAA CT	TGG TCA TGA GCC CTT CCA CA	83

Sometimes two sets of primers were used: #1 for standard RT-PCR (product sizes between 350 and 450 bp) and #2 for real-time PCR (product sizes between 80 and 150 bp). The housekeeping genes *GAPDH* and *Rpl-p0* were used for signal normalization in RT-PCR and real-time PCR, respectively.
Abbreviations: *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HSP*, heat shock protein; *IGFBP*, insulin-like growth factor-binding protein; *RPL-p0*, ribosomal protein, large, p0; RT-PCR, reverse transcription–polymerase chain reaction; *sFRP-2*, secreted frizzled-related protein 2.

Both Contact and Noncontact Cultures with UG26-1B6 and EL08-1D2 Support Hematopoietic Repopulating Cells

To determine the ability of six different cell lines to maintain the ability of K6-SP cells to repopulate mice, K6-SP cells were cultured on irradiated stromal cells. In the present series of experiments, we used K6-SP cells instead of $CD31^+$-K6 cells [8], and each culture contained approximately 30 repopulating cells instead of 3 to 6 in our previous work. The first colonies of proliferating cells appeared after 1–2 weeks of culture. These colonies proliferated rapidly thereafter. After 4 weeks of culture, complete cultures were harvested and transplanted into at least three irradiated recipients per culture. Six and 16 weeks after transplant, PCR analyses established that cocultures from both EL08-1D2 and UG26-1B6 contained cells with repopulating ability, both at 6 weeks (not shown) and 16 weeks after transplant, whereas cocultures with either AM20-1B4 or AM30-3F4 did not (Table 3). In all cultures, the frequency of repopulating cells decreased from 1 in 10 K6-SP cells to 1 in 19 to 25 input cells on EL08-1D2 and UG26-1B6 and below 1 in 85 to 125 for the aorta/mesenchyme-derived cells AM20-1B4 and AM30-3F4. Unexpectedly, we found that both EL08-1D2 and UG26-1B6 support the maintenance of repopulating ability when K6-SP was not in direct contact with the stromal cells (Table 3). In cultures on UG26-1B6, the frequency of repopulating cells was unchanged in contact and noncontact cultures (1 in 19 and 1 in 20, respectively). Interestingly, the frequency of repopulating cells in noncontact cultures on EL08-1D2 seems to remain higher (1 in 5 input K6-SP cells) compared with the direct contact cultures (1 in 25). These results demonstrate that HSCs need not be in direct contact with EL08-1D2 or UG26-1B6 to be maintained.

Macroarray Analysis of Gene Expression Patterns Between HSC-Supportive and Nonsupportive Stromal Cells

The above experiments suggest that HSC maintenance can be supported by soluble factors. What soluble growth factors are produced by embryonic stromal cell clones? Our earlier studies suggested that the chordin-like gene might be expressed at a higher level in HSC-supportive cell lines. Expression levels of other growth factors (TPO, SCF, FL, G-CSF, IL-1β, IL-6, IL-11, leukemia inhibitory factor, OSM, and transforming growth factor β [TGFβ]) were very similar between supportive and nonsupportive stromal cells [7].

To find more distinctive markers between HSC-supportive stromal cells and HSC-nonsupportive cells, we performed macroarray-based gene expression analyses. When single cell lines are compared with other cell lines, many differentially expressed cells may be found. These genes usually represent differences in cellular lineages but do not necessarily provide information about a certain function of the cell lines—that is, whether they can support HSCs. To minimize the possibility that only cell type–specific information would be obtained, we opted to compare the combination of both EL08-1D2 and UG26-1B6, which are of different cell lineage and origin, with four different cell lines derived from different embryonic regions that did not support repopulating cells in culture (Table 3) [8]. Our analysis (see Materials and Methods) of this combination strategy revealed 31 genes to be differentially expressed between HSC-supportive and HSC-nonsupportive cell lines. Twenty of these genes are expressed at a higher level in the two supportive cells (Table 4), and 11 genes were expressed at a lower level (Table 5). Genes with highest (more than threefold) expression differences were fibroblast growth factor-7 (FGF-7), cathepsin K, thrombospondin 2 (TSP2), pleiotrophin (PTN), and the IGFBP-3 and -4.

The differential expression of many of the genes found in the macroarray analyses (Tables 4 and 5) could be confirmed by RT-PCR (Fig. 2A), real-time RT-PCR (Fig. 2B), and, in some cases, ELISA (Fig. 3). Although PTN mRNA was expressed by both EL08-1D2 and UG26-1B6 as confirmed by RT-PCR and real-time PCR (Figs. 2A and 2B, respectively), we detected secretion of this 17-kDa heparin-binding protein only in the conditioned medium of EL08-1D2 (data not shown). Also, IGFBP-3 message was found in all cell lines by RT-PCR (not shown), reflecting the fact that all cell lines show a relatively high level of gene

Table 3. Maintenance of murine bone marrow K6-SP cells on embryonic stromal cells

	Contact cultures		Noncontact cultures	
4–6 weeks' culture on	**Positive/total**	**HSC frequency**	**Positive/total**	**HSC frequency**
UG26-1B6	9/22	1 in 19	2/5	1 in 20
EL08-1D2	7/21	1 in 25	6/7	1 in 5
AM20-1B4	0/8	<1 in 85	0/3	<1 in 35
AM30-3F4	0/12	<1 in 125	0/3	<1 in 35

Cocultures were initiated with 300 K6-SP cells from male Ln72 transgenic mice in direct contact or separated from each other by a 0.4-μm collagen-coated membrane (noncontact) with embryonic stromal cells. After 4 (two experiments) to 6 (one experiment) weeks, the complete cultures were harvested and transplanted into three lethally irradiated female B10CBA recipient mice together with 2×10^5 B10CBA spleen cells. After 16 weeks, peripheral blood was investigated for the presence of the transgenic human β-globin marker by semiquantitative polymerase chain reaction [8]. Recipient animals were classified as positive when more than 10% of peripheral blood cells were of Ln72 donor origin. The HSC frequency was estimated using the L-Calc statistical package v1.1 (StemCell Technologies).
Abbreviations: HSC, hematopoietic repopulating stem cell; SP, side population.

Table 4. Combined gene expression profile of EL08-1D2 and UG26-1B6 compared with the combined gene expression profile of UG15-1B7, EL28-1B3, AM20-1B4, and AM30-3F4: genes expressed at a higher level

Gene coordinate	GenBank accession number	Protein/gene	Array type	EL08-1D2	UG26-1B6	Mean EL08 and UG26	UG15-1B7	EL28-1B3	AM20-1B4	AM30-3F4	Mean UG15, EL28, AM20, and AM30	Log ratio	Fold change	*p* Value (*t*-test, two-sided)
D14b	X81582	IGFBP-4	M1.2	4.95	2.97	**3.96**	2.00	2.31	1.66	2.33	**2.07**	1.89	**6.60**	.04
D12c	D90225	Pleiotrophin (PTN. OSF-1)	M1.2	5.11	3.91	**4.51**	1.82	2.53	3.76	2.67	**2.69**	1.82	**6.14**	.01
D14a	X81581	IGFBP-3	M1.2	4.85	5.37	**5.11**	3.87	3.08	4.66	2.76	**3.59**	1.52	**4.57**	<.01
F04h	X94444	Cathepsin K	M1.2 II	6.39	6.14	**6.27**	4.71	5.24	5.04	4.66	**4.92**	1.35	**3.85**	<.01
B09g	L07803	Thrombospondin 2	M1.2 II	7.61	6.89	**7.25**	5.10	4.79	6.97	7.21	**6.02**	1.23	**3.43**	.03
D14f	Z22703	FGF-7 (KGF)	M1.2	4.51	4.10	**4.31**	1.83	2.29	4.10	4.44	**3.17**	1.14	**3.13**	.04
D13b	Z29532	Follistatin (FST; activin-binding protein)	M1.2	4.02	4.28	**4.15**	2.96	2.91	2.65	3.76	**3.07**	1.08	**2.94**	<.01
B01i	X83601	Pentaxin-related gene	M1.2 II	7.34	7.58	**7.46**	5.77	5.61	7.11	7.10	**6.40**	1.06	**2.89**	.01
D13m	X81585	IGFBP6	M1.2	4.84	3.98	**4.41**	3.26	4.27	3.18	2.71	**3.35**	1.05	**2.87**	.02
D13a	D38258	FGF-9	M1.2	2.99	3.00	**2.99**	2.01	2.10	1.95	1.87	**1.98**	1.01	**2.75**	<.01
D12b	S71251	CCL7 (MCP3)	M1.2	4.83	4.39	**4.61**	3.55	3.13	4.38	3.39	**3.61**	1.00	**2.72**	<.01
F07f	L07803	Thrombospondin 2	M1.2	4.24	3.91	**4.07**	2.77	2.60	3.11	3.81	**3.07**	1.00	**2.72**	<.01
A09j	U66918	SHOX2 (PRX3)	M1.2	3.19	3.38	**3.28**	2.55	2.30	2.21	2.20	**2.32**	0.97	**2.63**	<.01
D05j	AF039601	TGF-betaRIII (betaglycan)	M1.2	4.32	3.97	**4.14**	3.21	3.03	3.64	3.46	**3.33**	0.81	**2.25**	<.01
D03g	M20658	Interleukin-1 receptor	M1.2	3.69	3.50	**3.59**	2.95	2.47	2.79	2.99	**2.80**	0.80	**2.22**	.01
F14j	L23971	Fragile X mental retardation protein	M1.2	4.28	3.89	**4.09**	3.73	3.25	3.01	3.29	**3.32**	0.77	**2.15**	<.01
E04g	U33323	Preproglucagon	M1.2	2.12	2.40	**2.26**	1.38	1.78	1.40	1.44	**1.50**	0.76	**2.13**	.01
D11b	X80903	Delta-like protein 1 (DLL1)	M1.2	2.64	2.55	**2.59**	1.79	2.00	1.79	1.90	**1.87**	0.73	**2.07**	<.01
F07j	M87276	Thrombospondin 1	M1.2	5.11	5.42	**5.27**	4.98	3.84	4.25	5.13	**4.55**	0.71	**2.04**	.03
F03f	D67076	ADAM-TS1	M1.2 II	6.74	6.45	**6.59**	6.43	5.35	5.33	6.46	**5.89**	0.70	**2.01**	.05

Total RNA of confluent irradiated stromal cell lines was translated into [^{32}P]-labeled first-strand cDNA. After hybridizations with Atlas Mouse 1.2 and Mouse 1.2 II nylon arrays and measurement of hybridization signal, normalized signal intensities of the two cell line types (HSC-supportive and HSC-nonsupportive) were compared. For the comparisons, all arrays were transformed according to the method described by Huber et al. [13]. The data represent the mean of the normalized signal of the two hybridizations performed for each cell line. Only signals with a significant *p* value ($p < .05$, Student's *t*-test) and more than twofold relative over-representation are shown.
Abbreviations: FGF, fibroblast growth factor; HSC, hematopoietic repopulating stem cell; IGFBP, insulin-like growth factor-binding protein; TGF, transforming growth factor.

expression (Table 4). On the protein level, however, only EL08-1D2 and UG26-1B6 were found to secrete high levels of IGFBP-3, and AM20-1B4 secreted medium levels compared with the other cell lines, confirming the difference found in the macroarray analysis (Fig. 3). The array analyses additionally revealed that another IGFBP, IGFBP-4, was strongly expressed in EL08-1D2, whereas expression in UG26-1B6 was only slightly higher than in the other four stromal lines, and this was confirmed by real-time PCR (Fig. 2B). Other RT-PCR and real time-PCR experiments confirmed the relative overexpression of TSP2 and cathepsin K in EL08-1D2 and UG26-1B6. Follistatin was expressed in all cell lines, but the highest expression was found in EL08-1D2, UG26-1B6, and EL28-1B3. In addition, the soluble frizzled-related protein sFRP-2 (secreted frizzled-related protein 2) was confirmed to be overexpressed in cell lines, supporting repopulating activity by real-time PCR (Fig. 2B). Moreover, we show that GPX3 and HSP27 are relatively overexpressed in cell lines that do not support repopulating cells in culture (Figs. 2A, 2B).

Discussion

The main finding of the present work is that adult bone marrow HSCs do not require direct contact with embryonic stromal cells to be maintained in culture. We find that both EL08-1D2 and UG26-1B6 support the maintenance of K6-SP HSCs for more than 4 weeks. Thus, EL08-1D2 and UG26-1B6 differ from previously described supportive AGM-derived cell lines AGM-S3 and DAS104-4 [5, 6] and also from the E14 fetal liver–derived line AFT024 [14] in their ability to support hematopoietic cells in the absence of direct contact. In an attempt to find a distinctive molecular signature for HSC-supportive stromal cells, we found five genes to be differentially expressed by at least threefold between

Table 5. Combined gene expression profile of EL08-1D2 and UG26-1B6 compared with the combined gene expression profile of UG15-1B7 and EL28-1B3, AM20-1B4, and AM30-3F4: genes expressed at a lower level

Gene coordinate	GenBank accession number	Protein/gene	Array type	EL08-1D2	UG26-1B6	Mean EL08 and UG26	UG15-1B7	EL28-1B3	AM20-1B4	AM30-3F4	Mean UG15, EL28, AM20, and AM30	Log ratio	Fold change	*p* Value (*t*-test, two-sided)
C09h	U13705	Plasma glutathione peroxidase	M1.2	2.31	1.80	**2.05**	4.82	2.24	5.00	4.90	**4.24**	2.19	**8.92**	< .01
B14m	M74570	ADH family 1 subfamily A2	M1.2 II	6.33	5.46	**5.90**	6.93	7.79	6.91	7.22	**7.21**	1.31	**3.72**	.01
F14d	U77039	Four and a half LIM protein 1 (FHL1)	M1.2	1.63	1.73	**1.68**	1.99	3.55	2.07	3.17	**2.70**	1.02	**2.77**	.01
F08n	M13806	Cytokeratin 14	M1.2	1.95	2.03	**1.99**	2.47	4.11	2.70	2.49	**2.94**	0.95	**2.60**	.01
F14c	U26967	Cordon-blue protein (COBL)	M1.2	1.51	1.97	**1.74**	2.14	3.19	2.79	2.41	**2.63**	0.89	**2.44**	.01
D05l	Z18278	5-hydroxitryptamine (serotonin) receptor	M1.2 II	3.68	4.14	**3.91**	4.51	4.32	4.45	5.67	**4.74**	0.83	**2.29**	.03
C08l	U03560	HSP27	M1.2	2.85	2.79	**2.82**	3.89	4.19	3.12	3.25	**3.61**	0.79	**2.21**	< .01
A02i	X04836	CD4 receptor	M1.2	1.96	1.66	**1.81**	2.17	3.14	2.83	2.22	**2.59**	0.78	**2.19**	.03
D07j	AF035777	Somatostatin receptor 5	M1.2	1.77	1.29	**1.53**	1.94	2.25	2.60	2.30	**2.27**	0.74	**2.10**	.01
D08f	J04192	Cholinergic receptor, muscarinic	M1.2 II	3.82	4.18	**4.00**	4.89	4.39	4.61	4.98	**4.72**	0.72	**2.05**	.03
F09a	M11686	Cytokeratin 18	M1.2	1.75	1.99	**1.87**	1.97	3.53	2.45	2.36	**2.58**	0.71	**2.03**	.03

Total RNA of confluent irradiated stromal cell lines was translated into [^{32}P]-labeled first-strand cDNA. After hybridizations with Atlas Mouse 1.2 and Mouse 1.2 II nylon arrays and measurement of hybridization signal, normalized signal intensities of the two cell lines were compared. For the comparisons, all arrays were transformed according to the method described by Huber et al. [13]. The data represent the mean of the normalized signal of the two hybridizations performed for each cell line. Only signals with a significant p value ($p < .05$, Student's t-test) and more than twofold relative under-representation are shown.

the two HSC-supportive lines EL08-1D2 and UG26-1B6 and the four nonsupportive counterparts.

Several investigators have reported that stromal cells are necessary for the maintenance of bone marrow HSCs because they provide anchorage as well as growth factors [15]. The anchorage is for the most part mediated by β1 integrin's very late activation antigen (VLA)-4 and VLA-5 and their ligands' vascular cell adhesion molecule (VCAM)-1 and fibronectin, respectively. The β1 integrin-mediated signals are apparently aimed to prevent most early progenitors from entering the cell cycle [16]. Thus, we were surprised to find that maintenance of repopulating activity did not require the K6-SP cells to be in direct contact with either EL08-1D2 or UG26-1B6, embryonic cell lines we previously found to support HSC maintenance [8]. Moreover, we found that a key molecule involved in integrin-mediated adhesion and cycle regulation, VCAM-1, is expressed at a significantly lower level in both UG26-1B6 (4.2-fold lower expression compared with UG15-1B7) and EL08-1D2 (2.3-fold lower expression than in EL28-1B3, not shown) than in stromal cells, which do not support HSCs in culture. This finding suggests there is redundancy in the mechanisms that keep HSCs in a quiescent state, some of which require anchorage-mediated signals and some of which do not. Such mechanisms may involve other (integrin-mediated) adhesion pathways or involve soluble molecules.

Several different stromal cell lines have been described to support HSC maintenance in culture. It is plausible that the mechanism by which HSCs are maintained depends on similar mechanisms in such cell lines. Based on this assumption, it should be possible to describe a common molecular signature of stromal cells that support bone marrow HSCs in culture. In our comparison of the gene expression profile of two HSC-supportive (EL08-1D2 and UG26-1B6) and four HSC-nonsupportive (AM30-3F4, EL28-1B3, AM20-1B4, and UG15-1B7) cell lines, we thus focused on genes that might be involved in such a common mechanism of HSC maintenance. When we do so, we find 31 genes to be consistently differentially expressed more than twofold in the two HSC-supportive stromal cell lines compared with nonsupportive cells. It is interesting that approximately one third of the genes expressed at a higher level in HSC-supporting stromal cells are secretory proteins. This finding would favor the view that maintenance of HSCs is not supported by alternatives to VCAM-1–dependent adhesion pathways but by mechanisms depending on soluble molecules.

In this light it is interesting to note that K6-SP cells grown under noncontact conditions show a significantly higher stem cell activity than cells grown in direct contact with EL08-1D2. Although we can only speculate about the mechanisms involved, it seems that under contact conditions, EL08-1D2 maintains repopulating activity (a decrease of HSC frequency from 1 in 10 to 1 in 25; Table 3), whereas under noncontact conditions, slight

increase in repopulating cell number is observed (HSC frequency of 1 in 5; Table 3). Again, this would favor the view that HSC maintenance on embryonic AGM-derived stromal cells is supported by contact-independent mechanisms.

To find clues about which molecules are commonly involved in HSC maintenance, it is of interest to compare the gene expression analyses of different HSC-supportive cell lines (AFT024 [17], HS27a [18]). The comparison of our analysis of approximately 2,200 genes with other reported studies reveals some striking similarities. For instance, the HSC-supportive cell line AFT024 expresses high levels of PTN, a TSP family member, and IGFBP-3 [17]. Interestingly, IGFBP-3 was cloned as a differentially expressed molecule between hematopoiesis-supportive human brain–derived endothelial cell lines [19]. This insulin-like growth factor (IGF)–binding protein was also highly expressed on AFT024 and the long-term culture-supportive cell line HS27a [18]. A detailed comparison between different datasets should enable further pinpointing of genes required to be expressed in an HSC-supportive environment.

At present the exact role of the molecules we found to be differentially expressed between HSC-supportive cells and non-supportive cell lines for HSC maintenance is unknown. None of these molecules has been studied in detail in the context of HSC maintenance or expansion. PTN, for example, is a heparin-binding cytokine that in the embryo is expressed in metanephric mesenchymal cells [20] and the mesenchyme of the developing liver [21]. PTN is known as a guidance molecule for neurites and osteoblasts, probably by using surface-expressed syndecan 3 [22]. PTN knockout mice are viable and fertile and display enhanced long-term potentiation in hippocampal slices [23]. Whether PTN deficiency also affects HSCs has to date not been investigated in these mice. PTN binds to several cell-surface molecules, including syndecan 3 [22], anaplastic lymphoma kinase [24], protein tyrosine phosphatase receptor Z (also known as RPTP-β and PTP-ζ) [25], and cytoplasmic nucleolin [26]. Expression of nucleolin was demonstrated in HSCs [27]. Nucleolin acts like a shuttle between cytoplasm and nucleolus and may pick up endocytosed PTN and import PTN into the nuclear fraction. Whether PTN localizes in the nuclear fraction of HSCs or stromal cells is an issue we are addressing in a follow-up study.

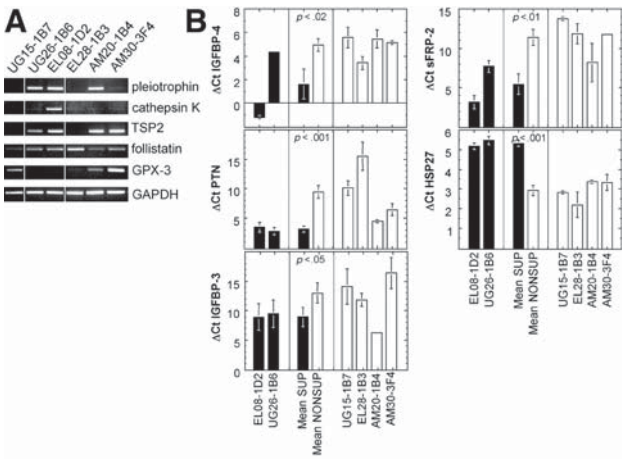

Figure 2. Confirmation of macroarray analyses at mRNA level. The expression of some candidate HSC regulatory genes (Tables 4 and 5) was determined by **(A)** reverse transcription-PCR **(B)** and real-time PCR. Total RNA was isolated, and cDNA prepared using poly-T primer from confluent irradiated stromal cell lines as described in Materials and Methods. cDNA, 0.5 μg, was then amplified using the primers described in Table 1 by PCR. In the representation of the real-time PCR, results of the cycle numbers required for a half-maximum response (Ct) was calculated as the difference (ΔCt) between the Cts of the housekeeping gene *RPL-p0* and that of the test molecule. The bars represent the mean and SE of the mean of three independent experiments (three different passages of the cell lines indicated). The black bars depict the HSC-supportive cell lines, and the white bars the cell lines that do not support maintenance of HSC in culture. The smaller the ΔCt, the higher the expression level. Negative ΔCt represents expression levels higher than the housekeeping gene (see for instance the level of IGFBP-4 in EL08-1D2). Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX-3, plasma glutathione peroxidase 3; HSC, hematopoietic repopulating stem cell; HSP27, heat shock protein 27; IGFBP, insulin-like growth factor-binding protein; PCR, polymerase chain reaction; PTN, pleiotrophin; RPL-p0, acid large ribosomal protein p0; sFRP-2, secreted frizzled-related protein 2; TSP2, thrombospondin 2.

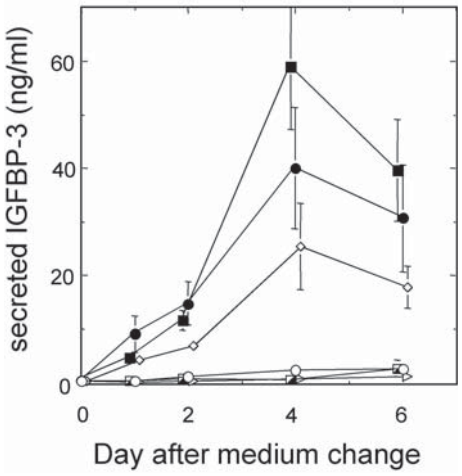

Figure 3. Confirmation of macroarray analyses at the protein level. The expression of insulin-like growth factor-binding protein (IGFBP-3) was determined by enzyme-linked immunosorbent assay (ELISA). Proteins were isolated from confluent, irradiated stromal cell lines as described in Materials and Methods. Data plot of ELISA analysis of the IGFBP-3 content in conditioned media collected from irradiated stromal cells at different time points after the first complete medium change. Shown is the analysis of conditioned media from EL08-1D2 (closed squares), UG26-1B6 (closed circles), UG15-1B7 (open circles), AM20-1B4 (open diamonds), EL28-1B3 (half-filled squares), and AM30-3F4 (open triangles).

IGFBP-3 and -4 are abundantly expressed binding proteins for IGFs. IGFBP-4, together with IL-6 and osteopontin (confirmed by ELISA; S.M. and R.A.J.O., unpublished results), is particularly strongly expressed in EL08-1D2, suggesting that this cell line might represent an osteoblastic cell. The activity of IGFBP-4 on hematopoietic cells has to date not been explored. IGFBP-3 is found at high levels in serum, where it forms a heterotrimeric complex with IGF and the acid-labile subunit. All three components are expressed by the cell lines we investigated. However, only IGFBP-3 is differentially expressed between HSC-supportive and nonsupportive cell lines. These findings suggest that IGFBP-3 could be one of the molecules commonly involved in the regulation of HSC behavior. Although IGFBP-3 binds IGF and modulates its availability, IGFBP-3 generates IGF-independent signaling. For instance, IGFBP-3 signals through an IGF receptor–independent pathway to phosphorylate Smad-2 and -3 and downregulate Smad-4 [28], which are involved in signaling through TGFβ family members. The responsible IGFBP-3-binding receptor, however, has not been identified as yet. A perhaps interesting feature of IGFBP-3 is that its sequence contains a nuclear localization sequence and is not only found as a secreted molecule but can also bind intranuclear target genes, including p53, and retinoic X receptor alpha (reviewed by Lee and Cohen [29]). Indeed, we have found IGFBP-3 in nuclear extracts of both EL08-1D2 and UG2-1B6 (S.M. and R.A.J.O., unpublished results). Thus, IGFBP-3 may affect HSC maintenance not only in its secreted form but also through its nuclear counterpart. We are currently investigating how IGFBP-3 and its localization affect HSC maintenance.

The functions of the remaining factors overexpressed in HSC-supportive cell lines Cathepsin K, TSP2, FGF-7, and pentaxin-related gene have not been investigated in relation to hematopoiesis. Cathepsin K is a cystein protease that is normally expressed by osteoclasts and that has been shown to play a role in bone resorption. Although one could postulate that the proteolytic activity of cathepsin K might be beneficial in the formation of the marrow niche, it is unclear what role it might have in the AGM region. The heparin-binding FGF-7 (also known as keratinocyte growth factor) is known to be a critical factor for epithelial stem cells. Its receptor FGFR2IIIb (also known as K-sam) is expressed by epithelial cells, but it is unknown whether HSCs express this receptor. Hematopoietic cell lines transduced with K-sam proliferate in response to FGF-7 [30], indicating the intracellular machinery necessary for FGF-7 signal transduction is present. TSP2 is an extracellular antiangiogenic as well as antiosteogenic matrix molecule. Mice deficient in TSP2 show increased proliferation of microvascular endothelial [31] and mesenchymal stem cells [32]. A recent report shows that megakaryocytes fail to thrive in a TSP2-deficient marrow environment, which indicates that TSP2 is required for megakaryocyte differentiation [33]. It may well be that HSCs also require interactions with TSP2 to be maintained in appropriate numbers. The pentaxin-related gene (also known as long pentraxin PTX3 and TSG-14) is an acute-phase protein that is expressed after inflammatory stimuli (including ischemia and microbial infection). PTX3-deficient mice are particularly susceptible to fungal infection [34].

In summary, our study shows that bone marrow HSCs do not require direct contact with E11-derived stromal cells to be maintained. Gene expression analysis of HSC-supportive stromal cells identified several soluble molecules that could be involved in bone marrow HSC maintenance. Although a limited number of genes were investigated, our data contribute to the definition of an HSC-supportive niche. Together with other such analyses [17, 18], common denominators of HSC support may be defined and investigated individually. We are presently developing retroviral vectors to study the effect of long-term knockdown of the expression of IGFBP-3 and -4, PTN, TSP-2, or FGF-7 in stromal cells to be used in cocultures testing HSC maintenance. In addition, gene expression profiles of stromal cells, which support HSCs in culture, should be combined with the stem cell gene profiling studies, which are in progress [27, 35, 36]. This could lead to the identification of novel ligand-receptor interactions with the potential to improve our understanding of the regulation of stem cell behavior by the microenvironment.

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