Mesenchymal lineage potentials of aorta-gonad-mesonephros stromal clones

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Background and Objectives. The characterization of stem cell microenvironments throughout ontogeny is of fundamental interest in the field of stem cell biology. Within the adult blood system, hematopoietic stem cells (HSC) are supported in the osteoblastic and endothelial bone marrow microenvironments. During mouse mid-gestation, the first HSC emerge autonomously in the aorta-gonad-mesonephros (AGM) region. However, little is known about this microenvironment. To study the cellular complexity of the AGM hematopoietic microenvironment and its relationship to HSC, we examined the potential of AGM stromal clones to differentiate into several mesenchymal lineages.

Design and Methods. Stromal cell clones from the mid-gestation mouse were cultured in appropriate conditions known to support osteogenic, adipogenic, chondrogenic and endothelial differentiation. Potentials of the stromal cells were scored by morphological examination of the cultures, specific staining and gene expression profile.

Results. We show that most clones possess uni/bilineage osteogenic, adipogenic and/or endothelial potential. The differentiation potential of the stromal clones appears to relate to their site of origin but not to their ability to support hematopoiesis. Moreover, we show that AGM HSC activity is unaffected by the osteogenic differentiation of UG26.1B6 stromal cells.

Interpretation and Conclusions. These results confirm the existence of mesenchymal stem/progenitor cells in the AGM region and suggest that the AGM hematopoietic microenvironment is highly complex, containing stromal cells with various mesenchymal lineage potentials.

Key words: microenvironment, AGM, hematopoietic stem cells, mesenchyme

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Hematopoietic stem cells (HSC) are at the basis of the adult hematopoietic system. These cells are able to self-renew and to differentiate into all blood cell types. In the adult bone marrow (BM), HSC are maintained in close association to stromal cells. These latter cells provide a supportive microenvironment for HSC called the HSC niche.[1,2] Since stromal cells are key regulators of HSC functions, studies on the molecular profile and cellular complexity of the HSC niche during development are under intensive consideration.[3-6] For example, osteoblasts have been shown to support human hematopoiesis[7] and to play a critical role in the BM HSC niche in adult mice.[6,8] More recently, through the identification of SLAM receptors differentially expressed by HSC, Kiel and co-workers elegantly revealed the existence of an *in vivo* endothelial HSC niche.[9] However, the role of such mesenchymal lineages has not as yet been examined in the embryonic hematopoietic microenvironment.

In the mouse embryo, the first definitive HSC are autonomously generated in the intra-embryonic aorta-gonad-mesonephros (AGM) region at embryonic day (E)10.5.[10,11] To better understand the role of hematopoietic microenvironment in this unique region, we previously isolated and characterized stromal cell lines from the AGM subregions (aorta mesenchyme and urogenital ridges) and also from the embryonic liver and gastrointestinal tract.[12] Phenotypic characterization places the AGM stromal clones in the vascular smooth muscle cells (VSMC) hierarchy in between a mesenchymal stem cell and a VSMC.[13] *In vivo* and *in vitro* assays clearly showed that some of the AGM stromal clones are potent supporters of hematopoietic progenitors and HSC when compared to adult BM and fetal liver cell lines.[14] Hence, the AGM cell lines can provide important signals for the maintenance of the first HSC.

It is known that the AGM region contains different types of mesenchymal stem/progenitor cells. Minasi *et al.* reported in the quail embryo a population of aorta-associated stem cells which contributes to blood, cartilage, bone and muscle tissues when transplanted into chick recipients.[15] We also have revealed a localization of mesenchymal stem/progenitor cells to the mouse AGM region and to the major hematopoietic tissues during mouse ontogeny. Interestingly, mesenchymal progenitors appear in the

AGM region at the time of HSC emergence,[16] suggesting a functional co-ordination between the mesenchymal and hematopoietic lineages.

Here, we took advantage of the previously isolated AGM stromal clones to study the cellular complexity of the AGM hematopoietic microenvironment and its relationships to HSC. We examined a panel of AGM stromal clones for multipotency to mesenchymal lineages.

Design and Methods

Animals

Mice were bred at the Erasmus University Medical Center according to institutional guidelines. Animal procedures were carried out in compliance with the Standards for Humane Care and Use of Laboratory Animals. Sca1-GFP transgenic mice[17] were used for isolation of BM HSC.

Stromal cell cultures

Maintenance of the stromal cell lines

Previously generated stromal cell lines produced from the AGM sub-regions (aorta-mesenchyme and urogenital ridges) and from the embryonic liver and gastrointestinal tract were used in the studies.[12,14] Most of these clones were derived from transgenic mice expressing the thermolabile form of the *SV40 Tag* gene under the control of β-actin or phosphoglycerate kinase promoters. The SV40 T antigen is active and has an immortalizing effect at the permissive temperature (33°C) but not at non-permisive temperatures (37°C and 39°C). We also used some stromal clones derived from *Ly6E-LacZ* transgenic mice. The *Ly6E-LacZ* transgene clones were derived at the same time as the *SV40 Tag* antigen stromal clones and provided a control for a non-immortalizing transgene. Stromal cells were cultured on 0.1% gelatin-coated dishes in stromal medium (50% M5300 long-term culture medium (StemCell Technologies), 35% α MEM medium, penicillin-steptomycin (Gibco), 15% fetal calf serum (FCS), Glutamax-I (Gibco) and 10 μM β-mercaptoethanol). Stromal cells were trypsinized and re-plated at a density of 1-2×10^4 cells/cm^2 one to two times per week. Cells were maintained at 33°C, 5% CO_2 in a humid atmosphere. MS-5 cells[18] (kindly provided by Dr L.Coulombel, INSERM, France) were cultured at 37°C in α MEM medium supplemented with 10% FCS.

Differentiation assays

To induce their differentiation, stromal cells were cultured at 37°C under appropriate conditions (*see below and the report by Mendes et al.*[16]) in 24- or 6-well plates (Costar) at a density of 1 to 2×10^4 cells/cm^2. The medium was refreshed every 3-4 days.

Osteogenic differentiation

Stromal cells were stimulated for 3-4 weeks in α MEM medium supplemented with 10% FCS, 10^{-4}M glycerophosphate (Sigma, St. Louis, MO, USA) and 0.2 mM L-ascorbic acid 2-phosphate (Sigma). For the alkaline phosphatase (ALP) staining, cells were washed in phosphate-buffered saline (PBS), fixed in paraformaldehyde 4% for 1 hour at 4°C and exposed for 30 minutes to the ALP naphthol AS-MX substrate (Sigma) according to the manufacturer's recommendations. Mineralization was scored by alizarin red staining (Sigma).

Adipogenic differentiation

Stromal cells were cultured for 1-2 weeks in α MEM medium supplemented with 10% FCS, 10^{-7}M dexamethasone (Sigma) and 100 ng/mL of insulin (Sigma). Adipogenic cultures were stained with oil-red-O solution (Sigma). Cells were fixed in 4% paraformaldehyde for 1 hour, washed in PBS and stained with oil red for 2 hours at room temperature.

Endothelial cultures

The ability of stromal cells to form vascular tubules was evaluated using matrigel assays (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations. Tubule-forming cells were dissociated by pipetting and the expression of endothelial markers was evaluated by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA, USA).

Chondrogenic differentiation

A micro-mass culture system was used.[19] Stromal cells were cultured in 15 mL conical tubes in a serum-free medium supplemented with 1x ITS (insulin-transferrin-selenium, Sigma), 1×10^{-4}M L-ascorbic acid 2-phosphate, 10^{-9} M dexamethasone and 10-100 ng/mL of transforming growth factor β1 (R&D Systems). After 3-4 weeks, the pellets were fixed in 4% paraformaldehyde for 1 hour, frozen, sectioned and stained with toluidine blue.

Hematopoietic assays

In vitro co-cultures

Adult BM hematopoietic cells enriched in stem cell activity were purified by cell sorting on the basis of *Ly6E-LacZ* transgene and ckit expression. The *GFP* reporter transgene is under the control of the Sca1 transcriptional regulatory elements.[17] Our previous studies showed that all BM and AGM HSC are within the GFP^+ sorted cell fraction.[17,20] Sorted cells (15-25×10^3 cells/well) were co-cultured on a monolayer of confluent irradiated stromal cells (30 Gy) in long-term medium (M5300, StemCell Technologies) with 1 μM of hydrocortisone. Co-cultures were maintained at 33°C, 5% CO_2 in a humid atmosphere. Half of the medium was changed after 1 week of culture. After 9-11 days of culture, cells

Table 1. Mesenchymal potential of embryonic stromal clones.

	Differentiation Potential				
	Osteogenic	*Adipogenic*	*Endothelial*	*Chondrogenic*	*HSC support*
UG26.1B6	+++	–	–	–	+++
UG26.3B5	++	+++	–	–	+
UG26.3D4	–	–	–	–	++
UG26.1B4	–	–	–	–	ND
UG26.2D3	++	–	–	–	+
UG07.1C6	–	+++	–	–	ND
UG15.1B7	–	–	ND	ND	–
AM20.1B4	–	–	++	–	–
AM30.3F4	+++	–	++	–	–
AM14.1C4	–	±	+	–	+
AM30.3F5	–	–	–	–	ND
GI29.2B4	++	–	++	–	+
EL08.1D2	–	±	–	–	+++
MS-5	–	+++	–	–	+++

Stromal cells generated from the AGM subregions (aorta plus mesenchyme (AM) and urogenital ridges (UG)), embryonic liver (EL) and gastrointestinal region (GI) were cultured under conditions inducing differentiation to osteogenic, adipogenic, endothelial and chondrogenic lineages. Differentiation potentials were scored by morphological examination of the cultures, gene expression analysis and the use of specific staining. The bone marrow-derived stromal cell line MS-5 was also included in this study. Plus signs indicate the presence and minus signs the absence of such potentials. ND: not determined. Clones derived from tsSV40 T antigen transgenic embryos include UG26.1B6, UG26.3B5, UG26.3D4, UG26.2D3, AM30.3F4, AM20.1B4, AM30.3F5 and GI29.2B4. Clones derived from Ly6E-lacZ transgenic embryos include UG07.1C6, UG15.1B7, AM14.1C4 and EL08.1D2. The HSC-supporting activity of the stromal clones has been reported in referenced articles[12,22,24,25] and by Durand et al. (submitted).

from both adherent and non-adherent fractions were counted and collected for flow cytometry analysis. In some experiments, stromal cells differentiated into osteoblasts were used. Stromal cells were exposed for 14 days to the osteogenic medium prior to the addition of hematopoietic cells.

AGM explant cultures

E11 AGM were dissected from wild-type embryos and cultured as explants in co-cultures with irradiated stromal cells. After 3 days at 37°C, the AGM explants were dissociated by collagenase treatment and single cell suspensions injected intravenously into irradiated recipients. Four months after transplantation, recipient peripheral blood DNA was analyzed by semi-quantitative polymerase chain reaction (PCR) for the donor cell marker *Ymt*. Recipients were considered positive only if more than 10% of DNA content of peripheral blood cells was of donor origin.

Phenotypic marker analysis

Cells were stained with phycoerythrin (PE) anti-Sca1, Flk1, ckit, Mac1, B220, CD41 and Ter119 antibodies (Pharmingen). In some cases, cells were stained with biotin-anti-CD31 or CD34 antibodies and then incubated for 15 min with streptavidin-PE. Between each step of labeling, cells were washed in PBS 10% FCS. Cells were stained with 7AAD (to exclude dead cells) during analysis on a FACScalibur (Becton Dickinson).

Reverse transcription-polymerase chain reaction analysis

Specific gene expression was analyzed by reverse transcription polymerization chain reaction (RT-PCR). Total RNA was extracted from confluent stromal cultures using Trizol (LifeTechnologies, Carlsbad, CA, USA). To avoid genomic DNA contamination, RNA samples were treated with RNAse-free DNAse I (Promega). After phenol/chloroform/isoamyl alcohol extraction and phenol precipitation, oligo-dT primers (Promega) and reverse transcriptase (SuperscriptII, Stratagene) were used for the cDNA synthesis: 1/40 of the reaction volume was used for each experiment with the following primers: β-actin, 5-'CCTGAACCCTAAGGCCAACCG-3' and 5'-GCTCATAGCTCTTCTCCAGGG-3'; osteocalcin, 5'-CTGACCTCACAGATCCCAAGC-3' and 5'-CTGTGACATCCATACTTGCAG-3'.

Results

Embryonic stromal cells differentiate into several mesenchymal lineages

To study embryonic stromal cell differentiation, we focused on 13 stromal clones previously isolated from various regions of *tsSV40 T antigen* (temperature sensitive) or *Ly6E-lacZ* transgenic mouse embryo (Table 1).[12] These stromal lines, derived from the urogenital ridges, aorta plus mesenchyme, embryonic liver or gastrointestinal tract (Table 1), have various hematopoietic supportive properties and are classified within the VSMC hierarchy.

Considering that some of the stromal lines used in this study express the *tsSV40* transgene (immortalizing activity at 33°C), we investigated whether this transgene affected cellular morphology, proliferation and apoptotic status upon temperature switch as compared to control *Ly6E-lacZ* transgenic stromal lines. Representative results are shown in Figure 1. When UG26.1B6 cells (*tsSV40* transgenic) were cultured at 33°C at a density of 2.5 to 5×10^3 cells/cm^2, confluent cell layers formed within 7-8 days (Figure 1A) and cell numbers increased 61-fold (Figure 1C). In contrast, when the same density of cells was cultured at 39°C the cells did not become confluent, lost their typical myofibroblastic morphology and increased only 4-fold, suggesting an arrest in proliferation (Figure 1A and C). The diminished proliferation at 39°C was not due to a temperature-dependent toxic effect, since the proliferation of UG07.1C6 (*Ly6E-lacZ* transgenic) cells occurred similarly at 33°C and 39°C (Figure 1C). Furthermore, as shown by annexin V staining, the majority of UG26.1B6 cells cultured for 7 days at 33°C (96%) or 39°C (93%) were viable, with only very few entering into apoptosis (Figure 1B). Forward and side scatter analysis confirmed that the cells undergo slight morphologic changes when

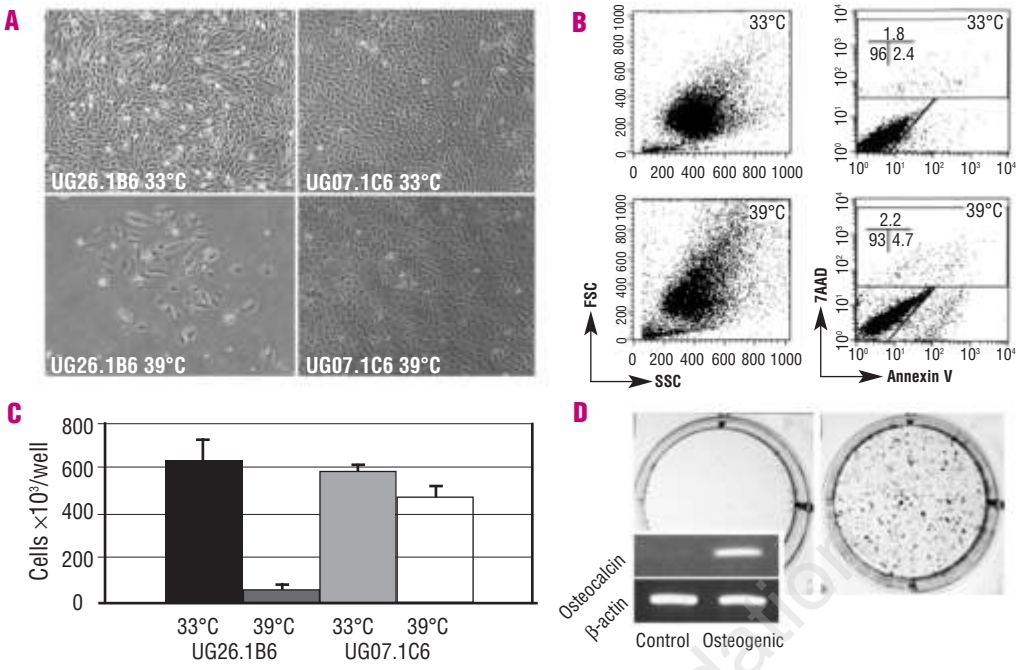

Figure 1. Effect of temperature on the proliferation, morphology and apoptotic status of the AGM-derived stromal clones. Two urogenital cell lines, UG26.1B6 (*tsSV40* transgene) and UG07.1C6 (*Ly6E-lacZ* transgene) are shown as examples. A. UG26.1B6 and UG07.1C6 cultures at 33°C and 39°C. B. Apoptotic status of UG26.1B6 cells cultured for 7 days at 33°C (top panels) and 39°C (bottom panels) and analyzed for forward (FSC)/side scatter (SSC) and annexin V-FITC and 7AAD staining. Slight morphological changes occurred at 39°C and cultures at 37°C also showed morphological and proliferative changes but with a temporal delay. C. Total cell number per well of UG26.1B6 and UG07.1C6 cells cultured for 8 days at 33°C and 39°C. Cells were plated at an initial density of 2.5 to 5×10³ cells/cm². Data are representative of the mean±SD of two independent sets of experiments conducted in triplicate. D. Kinetics of induction to the osteogenic lineage as detected by alkaline phosphatase activity. UG26.1B6 cells were induced to differentiate for 7 (left panel) and 14 days (right panel) at 37°C. RT-PCR analysis (inset) for osteocalcin expression in control (non-induced) or osteogenic UG26.1B6 cultures.

cultured at 39°C. Because differentiation processes usually require that cells exit from the cell cycle, we performed the differentiation analysis at 37°C, a non-permissive temperature.[19,21] These culture conditions were the same as those we had previously used to detect mesenchymal stem/progenitor cells in primary AGM tissues.[16] As shown by ALP activity and the expression of osteocalcin (Figure 1D), the osteogenic differentiation of UG26.1B6 cells occurs between day 7 and 14 of culture at 37°C. In addition, we observed that the osteogenic differentiation potential of the stromal clone was also efficient at the permissive temperature of 33°C.

Table 1 shows a summary of the mesenchymal differentiation potentials exhibited by the panel of stromal clones. UG26.1B6 cells demonstrated the most evident characteristics of osteogenic differentiation. After one week, UG26.1B6 cells started to form nodules (Figure 2A). From day 12 onwards, patches of cells and then nodules containing high ALP activity, a marker of early osteogenic differentiation (Figure 2B and C), were increased and peaked in number on day 14. On average, 70% of UG26.1B6 cells were ALP-positive at this time point (Figure 1D). Non-induced cultures were negative (*data not shown*). Mineralization, scored by strong alizarin red staining, was found in discrete osteogenic nodules from day 21-28 of culture (Figure 2D). RT-PCR analysis for osteocalcin expression also confirmed the osteogenic differentiation of UG26.1B6 (Figure 1D). Similar osteogenic differentiation was observed for UG26.2D3 and UG26.3B5. Except for high ALP activity of osteogenic differentiated AM30.3F4 and GI29.2B4 cells, none of the stromal clones from other anatomical sites was found to differentiate along this lineage.

Adipogenic potential was found in only two of the stromal cell clones tested. After 1 week of culture, UG26.3B5 and UG07.1C6 and the control BM MS-5 stromal clones showed abundant cytoplasmic lipid droplets typical of adipocytes. Representative undifferentiated (Figure 2E) and differentiated (Figure 2F and H) morphology and oil red staining (Figure 2G) are shown for UG26.3B5 and UG07.1C6. Endothelial potential was tested by culturing cells in matrigel. None of the urogenital ridge cell lines or EL08.1D2 was found to form vascular tubules. However, GI29.2B4 (Figure 2I) and several aorta plus mesenchyme-derived cell lines (AM20.1B4 in Figure 2J) homogeneously formed long tubules. To confirm the endothelial potential, cells were collected from matrigel cultures and analyzed for the expression of endothelial markers by flow cytometry. This technique showed that 11.6% of the GI29.2B4 and 4.4% of the AM20.1B4 cells expressed Flk1 (Figure 2K and L, respectively) after 1 day of culture. Finally, when cultured under chondrogenic conditions routinely used in

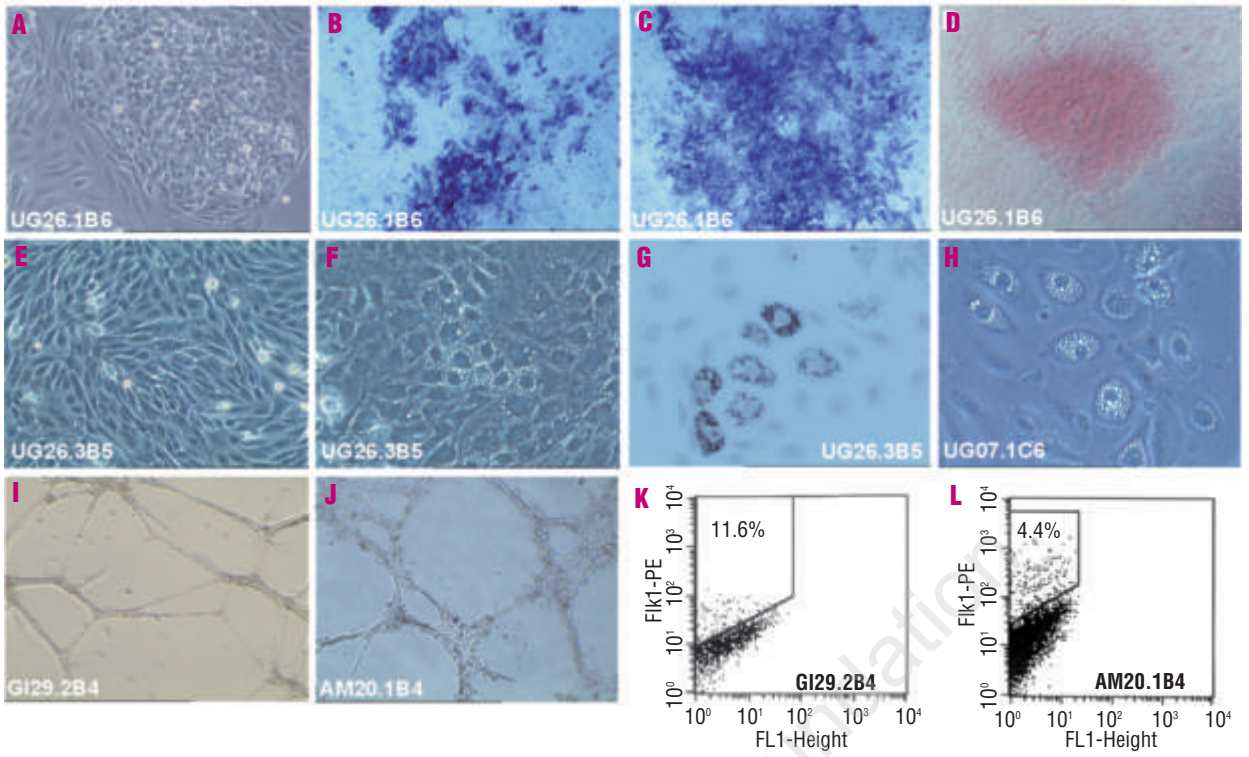

Figure 2. Mesenchymal potential of representative embryonic stromal clones. A-D. Osteogenic potential. UG26.1B6 cultured in a medium supplemented with ascorbic acid and β-glycerophophate show characteristics of osteogenic lineage differentiation: A. nodule formation, B and C. High ALP activity and D. calcium phosphate deposit as shown by alizarin red staining. E,H. Adipogenic potential. E. undifferentiated UG26.3B5, F. Fat droplet morphology of UG26.3B5 after adipogenic differentiation G. UG26.3B5 and H. UG07.1C6 stained with oil-red. I,L. Endothelial potential. I and J. GI29.2B4 and AM20.1B4 cells cultured in matrigel form long tubules. K,L. Flow cytometry analysis for the expression of Flk1 after 1 day of matrigel culture.

Table 2. *In vivo* long-term adult repopulation by AGM cells cultured in the presence of irradiated stromal cells.

Cell line	*N. of mice repopulated/transplanted*	*% of repopulated mice*
No line	5/16	31
UG26.3B5	3/16	18
UG26.1B6	5/12	42
UG26.1B6 osteogenic	6/14	43

AGM were dissected from wild-type day 11 male embryos and cultured as explants for 3 days in the absence or presence of a monolayer of UG26.3B5 and UG26.1B6 stromal cells undifferentiated or differentiated into osteoblasts. After 3 days of explant culture, AGM were collected and 0.3 embryo equivalents of cells were injected into irradiated female recipients. At 4 months post-transplantation engraftment levels ranged from 10-100%. Mice were considered engrafted if at least 10% of the blood cells were positive for the male donor genetic marker (Ymt). Data are from a total of four independent experiments.

our laboratory,[16] none of the 13 stromal clones was found to differentiate along this lineage pathway. Thus, most stromal clones appear to have uni/bi-lineage differentiation potential and the specific lineage differentiation potential may be related to the site of anatomical origin, with urogenital ridge lines most restricted to osteogenic and/or adipogenic differentiation and aorta plus mesenchyme lines to endothelial differentiation.

Differentiated stromal cells continue to support hematopoiesis

To determine whether the differentiation of stromal cells influences their ability to support haematopoiesis, we compared the hematopoietic supporting activity of undifferentiated and osteogenic differentiated UG26.1B6 cells. AGM tissues were co-cultured as explants on a filter above a confluent monolayer of irradiated undifferentiated or osteogenic-differentiated UG26.1B6 or control undifferentiated UG26.3B5 stromal cells (a non-supportive stromal clone).[14] After 3 days, AGM explants were recovered and 0.3 embryo equivalents of cells were injected into irradiated adult female mice. Recipient mice were analyzed for donor type (male) reconstitution at 4 months after transplantation (Table 2). AGM explants co-cultured with UG26.1B6 cells undifferentiated or differentiated into osteoblasts repopulated similarly, with 42% and 43% recipients highly engrafted (10-100% donor cell contribution).

Whereas co-culture with UG26.3B5 appears to have a negative effect on AGM HSC activity, osteogenic differentiation of UG26.1B6 does not affect its HSC supportive capacity.

We also evaluated the ability of differentiated stromal cells to support the differentiation of immature hemato-

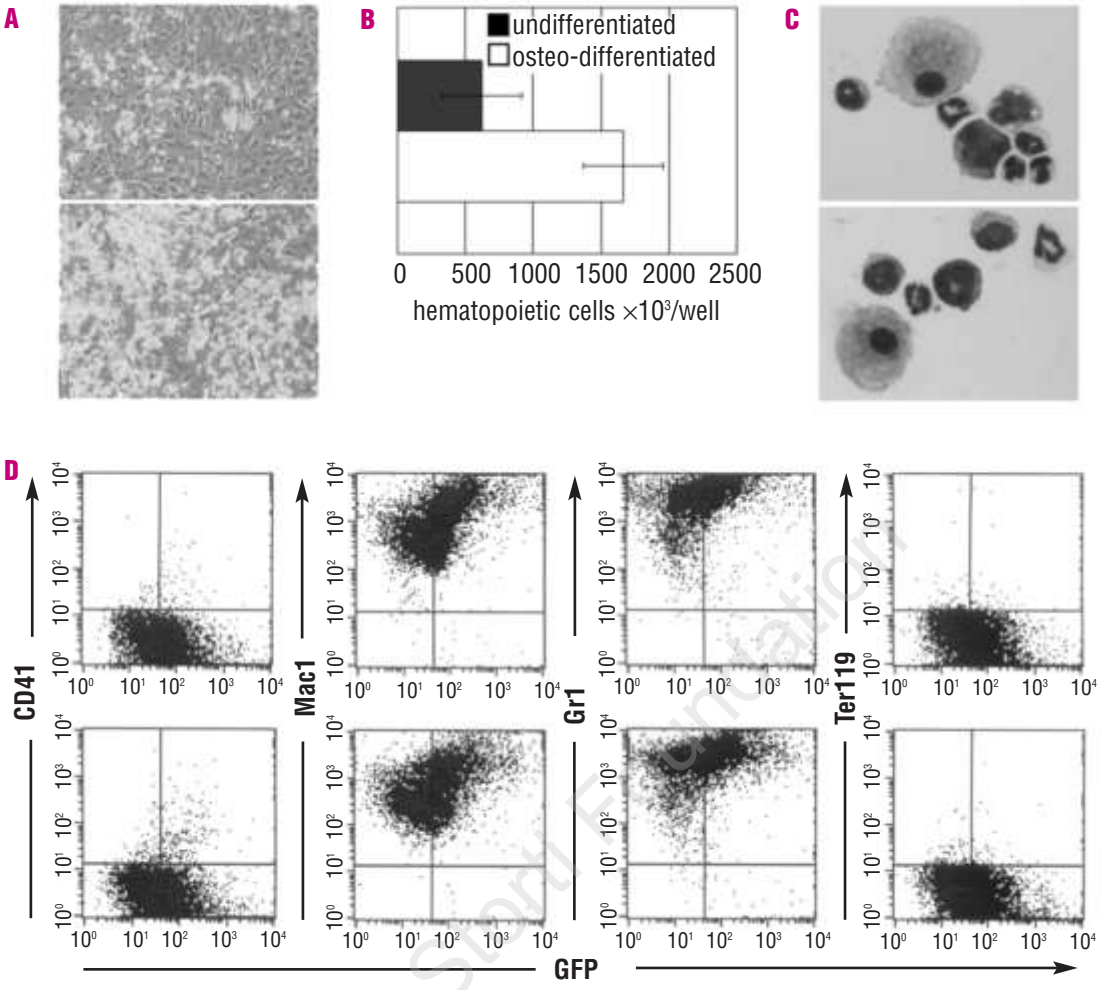

Figure 3. Co-cultures of adult BM c-kit⁺Sca-1GFP⁺ cells with UG26.1B6 stromal cells undifferentiated or differentiated into osteocytes. BM cells enriched in stem cell activity were purified by cell sorting on the basis of c-kit and Sca-1GFP expression and cultured for 9-11 days with irradiated stromal cells in the absence of cytokines (n=4). To induce the differentiation of UG26.1B6 cells, cells were cultured for 14 days in the osteogenic medium before the addition of BM hematopoietic cells. **A.** Representative pictures of hematopoietic cell growth in UG26.1B6 undifferentiated (top panel) and UG26.1B6 osteo-differentiated co-cultures (bottom panel). **B.** Total numbers of non-adherent hematopoietic cells per well in UG26.1B6 undifferentiated and UG26.1B6 osteo-differentiated co-cultures. The data shown represent the mean±SD. **C.** May Grünwald Giemsa-stained cytospins of non-adherent cells from UG26.1B6 undifferentiated (top panel) and UG26.1B6 osteo-differentiated co-cultures (bottom panel). **D.** Non-adherent cells from UG26.1B6 undifferentiated (top panels) and UG26.1B6 osteo-differentiated (bottom panels) co-cultures were analyzed by flow cytometry for the expression of GFP and mature lineage markers: CD41 for megakaryocytes, Mac1 for macrophages, Gr-1 for granulocytes and Ter119 for erythrocytes. Quadrants for positive and negative cells were determined by staining with appropriate isotype Ig control.

poietic cells from adult BM. ckit+Sca1GFP+ cells (enriched in HSC activity) were co-cultured for 9-11 days on monolayers of irradiated undifferentiated or osteogenic-differentiated UG26.1B6 stromal cells. Both undifferentiated and differentiated stromal cells supported the survival of hematopoietic cells, whereas cultures without stroma did not (90% of the c-kit+Sca-1GFP+ cells were dead by the end of the culture period (*data not shown*). Interestingly, the number of non-adherent cells was significantly higher (2.8-fold) in osteogenic UG26.1B6 than in the undifferentiated co-cultures (Figures 3A and B). Cytospin preparations of the non-adherent cells from both UG26.1B6 co-cultures revealed the presence of neutrophils, macrophages and megakaryocytes (Figure 3C). Adherent and non-adherent cell fractions were analyzed by flow cytometry for GFP and mature lineage marker expression. Flow cytometric analysis confirmed the presence of differentiated hematopoietic cells in both fractions (Figure 3D). Thus, osteogenic UG26.1B6 cells continue to support the survival, proliferation and differentiation of hematopoietic cells into myeloid lineages.

Discussion

A panel of AGM stromal clones was used to evaluate the cellular complexity of the AGM microenvironment and its relationships to HSC. We have shown that AGM

stromal clones, phenotypically near the end of the VSMC differentiation hierarchy (smooth muscle myosin heavy chain expression), are not definitively committed. While not exhibiting the full potency of mesenchymal stem cells, most clones possess uni/bi-lineage osteogenic, adipogenic and/or endothelial potential. Although none exhibited chondrogenic potential, this is not surprising since we found only a very low frequency of chondrogenic progenitors in primary E11 AGM differentiation cultures.[16] Additionally, we tested the mesenchymal differentiation potential of the BM-derived stromal line MS-5 that occupies a position early in the VSMC lineage.[13]

We found that MS-5 could differentiate only into adipocytes (Table 1). Thus, while stromal cell lines in various stages of VSMC differentiation still retain potency for other mesenchymal lineages, the differentiation potentials of stromal cells cannot be strictly correlated to the phenotypic position in the hierarchy. Additionally, when we compared the differentiation potential of the panel of the 13 clones with their ability to support hematopoietic progenitor/stem cells in long-term cultures (as determined in our previous studies[14]), no correlation was found between the differentiation potential and the hematopoietic-supportive ability.

As one of the most potent HSC supportive AGM stromal clones, UG26.1B6, normally expresses a wide variety of hematopoietic growth factors (e.g. IL-6, TGF-β, M-CSF).[12,22] Many of these molecules continue to be expressed after UG26.1B6 cells undergo osteogenic differentiation (*data not shown*). Since osteoblasts play an important role in hematopoiesis by producing granulocyte colony-stimulating factors and by interacting with BM HSC through the Notch signaling pathway,[6-8] it could be predicted that osteogenic differentiation would not interfere with the ability of UG26.1B6 to support hematopoiesis. Indeed, the differentiation of UG26.1B6 into the osteogenic lineage did not alter its HSC-supportive ability. In addition, in co-cultures, osteogenic-differentiated UG26.1B6 cells continued to strongly support hematopoietic differentiation of adult BM enriched HSC into megakaryocytes, macrophages and granulocytes. These data are consistent with the findings of Arai and colleagues.[23] These investigators identified a population of multipotent mesenchymal progenitors in the fetal limb bud which, when differentiated into osteocytes, are efficient in supporting the hematopoietic differentiation of adult BM c-kit+Sca-1+Lin- cells.

In conclusion, AGM stromal clones show a distribution of mesenchymal potentials. Hematopoietic supportive properties of the UG26.1B6 clone are not altered by differentiation to the osteogenic lineage. Together with the existence of primary mesenchymal stem/progenitor cells in the AGM,[15,16] our results showing the mesenchymal potential of AGM stromal lines support the notion that the AGM hematopoietic niche is highly complex. Future studies will investigate whether adipogenic-differentiated AGM stromal clones also support hematopoiesis. These stromal cells should aid in the future identification of the specific interactions in the embryo that lead to HSC growth.

All authors contributed to the design and analysis of the data and to drafting and revising the article. C. Durand performed the majority of the experiments, C. Robin perfomed some of the experiments and E. Dzierzak is head of the laboratory. All authors approved the final version of the manuscript.

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