Mesenchymal progenitor cells localize within hematopoietic sites throughout ontogeny

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Summary

Mesenchymal stem cells (MSCs) have great clinical potential for the replacement and regeneration of diseased or damaged tissue. They are especially important in the production of the hematopoietic microenvironment, which regulates the maintenance and differentiation of hematopoietic stem cells (HSCs). In the adult, MSCs and their differentiating progeny are found predominantly in the bone marrow (BM). However, it is as yet unknown in which embryonic tissues MSCs reside and whether there is a localized association of these cells within hematopoietic sites during development. To investigate the embryonic origins of these cells, we performed anatomical mapping and frequency analysis of mesenchymal progenitors at several stages of mouse ontogeny. We report here the presence of mesenchymal progenitors, with the potential to differentiate into cells of the osteogenic, adipogenic and chondrogenic lineages, in most of the sites harboring hematopoietic cells. They first appear in the aorta-gonad-mesonephros (AGM) region at the time of HSC emergence. However, at this developmental stage, their presence is independent of HSC activity. They increase numerically during development to a plateau level found in adult BM. Additionally, mesenchymal progenitors are found in the embryonic circulation. Taken together, these data show a co-localization of mesenchymal progenitor/stem cells to the major hematopoietic territories, suggesting that, as development proceeds, mesenchymal progenitors expand within these potent hematopoietic sites.

Key words: Mesenchyme, Hematopoiesis, AGM, Development, Embryo

Introduction

Mesenchymal stem cells (MSCs) have long been recognized for their ability to differentiate into cells of different connective tissue lineages, including bone, cartilage and adipose tissue, and therefore are of interest for potential therapeutic strategies. MSCs were first identified by Friedenstein, who reported an undifferentiated cell population within the adult bone marrow (BM) (Friedenstein et al., 1974). This population, under the appropriate stimuli, could differentiate into several mesenchymal cell types (Friedenstein et al., 1974). It is now well established, by both culture and in vivo studies, that BM-derived MSCs possess the ability to differentiate along osteogenic, chondrogenic and adipogenic lineages (Dennis et al., 1999; Horwitz et al., 1999; Jiang et al., 2002; Kuznetsov et al., 1997; Liechty et al., 2000; Pereira et al., 1998; Pittenger et al., 1999; Prockop, 1997; Sekiya et al., 2002). In addition to generating cells of several connective tissue lineages, MSCs also give rise to the hematopoietic supportive stroma (Dennis and Charbord, 2002; Devine and Hoffman, 2000; Prockop, 1997) that constitutes the hematopoietic microenvironment in adult BM. Indeed, several BM-derived stromal cell lines promote survival, proliferation and differentiation of hematopoietic stem and progenitor cells in vitro (Dennis and Charbord, 2002; Deryugina et al., 1994; Dexter et al., 1977; Gartner and Kaplan, 1980; Moore et al., 1997). Moreover,

MSCs enhance the engraftment of donor hematopoietic stem cells (HSCs) after co-transplantation in recipient animals (Almeida-Porada et al., 2000; Anklesaria et al., 1987). The ability of MSCs to regulate hematopoiesis both in culture and in vivo is thought to be mediated by both cell-to-cell contact and the production of growth factors involved in the homing, proliferation and differentiation of hematopoietic cells (Blazsek et al., 2000; Dormady et al., 2001; Quesenberry et al., 1993; Verfaillie, 1998). Recent reports show that osteoblastic cells function as a key component of the HSC-supportive BM microenvironment (Calvi et al., 2003; Zhang et al., 2003). Zhang et al. showed that the number of BM osteoblastic cells that are responsive to bone morphogenetic protein directly correlates with the number of BM HSCs (Zhang et al., 2003), and Calvi et al. demonstrated that parathyroid hormone-activated osteoblastic cells lead to an increase in HSC numbers through Notch signaling (Calvi et al., 2003). Adipogenic cells within the BM stroma may also be important for the maintenance of multipotent hematopoietic progenitors (Dexter et al., 1977). Despite our increasing understanding of the mesenchymal lineages involved in support of HSC growth in the adult BM, little is known of the mesenchymal cells supporting HSCs in the embryo.

During mouse ontogeny, the first adult-type HSCs are generated at E10.5 in the aorta-gonad-mesonephros (AGM) region (Medvinsky and Dzierzak, 1996; Muller et al., 1994).

Initially, HSCs are restricted to the regions of major vasculature: the vitelline and umbilical arteries and the dorsal aorta (de Bruijn et al., 2000). One day after their emergence in the AGM region, HSCs are also found in the other subregion of the AGM, the urogenital ridges, and also in the yolk sac (YS) and fetal liver (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Between E10.5 and E12.0, HSCs amplify within the AGM region and, later, these cells are thought to migrate and colonize the fetal liver, where they undergo extensive expansion to the numbers found in the adult (Kumaravelu et al., 2002). Finally, just before birth, HSCs are thought to migrate to the BM, which remains the main site of hematopoiesis throughout adult life (Cumano and Godin, 2001). Thus, HSC supportive microenvironments, presumably of mesenchymal origin, exist in several anatomical sites during ontogeny, the AGM, fetal liver and BM.

Recently, an early mesodermal precursor has been identified in the E9.5 mouse dorsal aorta. When transplanted into chick embryos, these cells participated in the development of vasculature and were integrated into blood, bone, cartilage and muscle (Minasi et al., 2002). Moreover, clonal progeny from an aorta cell line was able to differentiate in vitro into cells of hematopoietic, osteogenic, adipogenic and myogenic lineages (Minasi et al., 2002). Other reports demonstrate that cells from mouse embryonic dorsal aorta and fetal liver can differentiate in vivo along the myogenic lineage (De Angelis et al., 1999; Fukada et al., 2002). Hence, during midgestation, precursor cells for several mesenchymal lineages can be found within hematopoietic sites, but it is unknown whether there is a specific localization of such precursors to these sites. Moreover, the results of Blazsek et al. suggest that, during development, acquisition of hematopoietic competence by the BM is correlated with the emergence of cell aggregates (hematon units) composed of mesenchymal, endothelial and hematopoietic cells, as well as hematopoietic progenitors (Blazsek et al., 2000). These studies raise a number of questions. Where do mesenchymal stem/progenitor cells first emerge in the embryo? How are they distributed during ontogeny? Is their temporal and spatial distribution pattern effectively correlated with hematopoietic territories? And finally, is the presence of MSCs dependent on HSC activity?

We carried out a comprehensive anatomical mapping and frequency analysis of mesenchymal progenitors during ontogeny. The data presented here show that AGM, fetal liver, and neonatal and adult BM harbor stem/progenitor cells for several mesenchymal lineages. The presence of these cells in circulating blood suggests that at least part of these progenitors can migrate between tissues. Moreover, the progressive increase in numbers of mesenchymal progenitor/ stem cells throughout development indicates that these hematopoietic sites provide a microenvironment that supports the generation, maintenance and/or proliferation of mesenchymal stem/progenitor cells. Additionally, in E11 Runx1 deficient embryos, the presence, differentiation potential and frequency of these cells is not dependent upon HSC activity. Thus, our results show that mesenchymal progenitor/stem cells develop within the major hematopoietic sites throughout ontogeny, raising the possibility of a parallel and coordinate development of both hematopoietic and mesenchymal systems.

Materials and methods

Mice and embryo generation

Wild-type (C57BL/10×CBA) F1 mice were used for spatial and temporal mapping experiments. Matings for embryo generation were timed and the day of vaginal plug detection was designated as embryonic day 0 (E0). $Runx1^{-/-}$ embryos (used to evaluate the influence of HSCs on the differentiation potential and/or frequency of mesenchymal progenitors) were obtained by mating $Runx1^{+/-}$ (C57BL/6 background) mice (Cai et al., 2000). Oct4 GFP mice were a kind gift from K. Ohbo and K. Abe (Yoshimizu et al., 1999).

Tissues and cell preparations

YS, head, somites, limb buds, heart, liver, umbilical and vitelline arteries, and AGM region were dissected from E11 mouse embryos (Fig. 1A). Some AGMs were longitudinally sub-dissected, separating the dorsal aorta with its surrounding mesenchyme (AoM) from the gonads and mesonephros (urogenital ridges; UGRs) (Fig. 1B). All tissues were incubated for one hour at 37°C with 0.125% collagenase in phosphate buffered saline solution (PBS) containing 10% fetal calf serum (FCS) and penicillin/streptomycin (P/S). Primary cells were then dispersed, washed, resuspended in PBS with 10% FCS and P/S, and used in differentiation assays and frequency analysis. Hematopoietic tissues at other time points were isolated and tested: E12 YS, AGM and liver; E14 liver; neonatal (1 week old) and adult (12-14 weeks old) BM. After collagenase digestion, E14 liver cells were submitted to a ficoll gradient to deplete erythrocytes. Femora were dissected from surrounding tissues and BM cells flushed using a 25G needle. Cells were dispersed, resuspended in PBS with 10% FCS and P/S, passed through a nylon mesh filter. Blood was obtained at E11, E12, E14 and E17. Embryos were isolated with yolk sac intact, washed and placed in a culture dish containing PBS+10% FCS. Under the dissection microscope, the vitelline and umbilical arteries were cut at the base of the yolk sac allowing the blood to flow freely into the medium. The medium was collected and centrifuged, nucleated cells counts performed, and blood cells directly cultured.

Differentiation assays

Osteogenic differentiation

Primary cells from the several tissues were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 15% FCS, P/S, 0.2 mM L-ascorbic acid 2-phosphate and 0.01 M β-glycerophosphate. After 10 to 12 days, the cultures were analyzed for alkaline phosphatase (ALP) activity, as well as for gene expression of *Cbfa1* (*Runx2* – Mouse Genome Informatics), osteopontin (*Spp1* – Mouse Genome Informatics) and osteocalcin. Mineralization capacity was evaluated at day 21 by Alizarin Red staining.

Adipogenic differentiation

Primary cells from the several tissues were cultured for 2 to 3 days in DMEM with 10% FCS and P/S, and then treated in adipogenic medium consisting of DMEM, 1% FCS, P/S, 10^{-7} M dexamethasone and 100 ng/ml insulin. Seven to 10 days after stimulation, the cultures were evaluated based on morphology and *Pparg* gene expression.

Chondrogenic differentiation

A micro-mass culture system was used (Dennis et al., 1999). Primary cells from the several tissues were placed in polypropylene tubes, centrifuged to form a micro-mass and cultured for 21 days in DMEM containing ITS$^+$ (insulin-transferrin-selenium), P/S, 0.1 mM L-ascorbic acid 2-phosphate, 10^{-9} M dexamethasone and 20 ng/ml TGFβ1. After 21 days, tissue aggregates were frozen, sectioned and either stained with Toluidine Blue (which detects proteoglycans in the extracellular matrix), or fixed and subsequently stained with a collagen type II specific antibody (CIIC1, Developmental Studies Hybridoma Bank, Iowa). Secondary antibody, anti-mouse

immunoglobulin-HRP (Dako, Denmark) and a DAB substrate for peroxidase (Dako, USA) were used.

Frequency analysis

To estimate the frequency of osteogenic, adipogenic and chondrogenic progenitors present in the several hematopoietic tissues throughout development, primary cells of each tissue were tested in colony forming unit (CFU) assays (Friedenstein et al., 1974; Owen et al., 1987; Sekiya et al., 2002; Simmons and Torok-Storb, 1991) in association with the above mentioned differentiation assays. To determine the frequency of osteogenic and adipogenic progenitors, the minimum cell-seeding density at which cells grow in distinct colonies was determined for each tissue type (Table 1). With regard to the chondrogenic lineage, the minimum cell input for the formation of a tissue aggregate (or pellet) in micro-mass cultures was also determined (Table 1).

Osteogenic progenitors

After 10 to 12 days of stimulation in osteogenic differentiation medium, frequency was calculated as the number of ALP-positive colonies (D'Ippolito et al., 1999; Owen et al., 1987) divided by the input cell number.

Adipogenic progenitors

After 7 to 10 days of treatment, frequency was taken as the number of adipogenic colonies (Sekiya et al., 2002) divided by the input cell number.

Chondrogenic progenitors

After 21 days of culture in chondrogenic differentiation medium, frequency was determined by the number of distinct cartilage foci (Fig. 2E) divided by the input cell number. In this calculation, we assume that each foci in the micromass cultures would develop from at least one chondrogenic progenitor. Results from experiments with mixes of phenotypically marked cells indicate that approximately 80% of foci are most likely clonally-derived (S.C.M., unpublished). Thus, our indicated values may represent a slight underestimation of chondrogenic progenitors.

Statistical analysis

Results regarding progenitor frequency are expressed as the mean±s.d. Statistical evaluation was performed using single factor analysis of variance (ANOVA) to assess statistical significance between more than two groups. In addition, Student's t-tests were used to evaluate statistical differences between each two groups. Statistical significance was defined as $P<0.05$.

Gene expression

Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to determine the expression of osteocalcin and *Pparg* in both freshly isolated cells and cultures stimulated towards differentiation. Target genes were amplified by PCR with the following primers:

mouse *Cbfa1*, 5′-ACCACAGAACCACAAGTGCGG-3′ and 5′-CTGAAGAGGCTGTTTGACGC-3′;

mouse osteopontin, 5′-CTATAGCCACATGGCCGG-3′ and 5′-GAGGTCCTCATCTGTGGC-3′;

mouse osteocalcin, 5′-CTGACCTCACAGATCCCAAGC-3′ and 5′-CTGTGACATCCATACTTGCAG-3′;

mouse *Pparg*, 5′-GACCTCTCCGTGATGGAA-3′ and 5′-GCT-GGTCGATATCACTGG-3′; and

the house-keeping gene mouse β actin, 5′-CCTGAACCCTAA-GGCCAACCG-3′ and 5′-GCTCATAGCTCTTCTCCAGGG-3′.

Results

Anatomical mapping and differentiation potential of mesenchymal progenitors in embryonic tissues at midgestational stages

To test for the presence of mesenchymal stem/progenitor cells in the midgestational mouse embryo, cells from several tissues were isolated and cultured in media known to induce the differentiation of such cells into osteogenic, adipogenic and chondrogenic lineages (Fig. 1A). Embryonic day 11 (E11) YS, umbilical and vitelline arteries, AGM, head, somites (divided into differentiating somites, epithelial somites and presomitic mesoderm, including caudal somites), limb buds, heart and liver tissues were analyzed. Table 2 summarizes the results. At E11, with the exception of the AGM, somites and limb buds, no other tissues contained cells able to differentiate into any of the mesenchymal lineages tested.

Mesenchymal stem/progenitor cells for all three lineages were exclusively present in the AGM region. When cultured in osteogenic-inductive conditions, AGM cells formed colonies expressing alkaline phosphatase (ALP, Fig. 2A), a widely recognized marker of osteogenic activity (D'Ippolito et al., 1999; Oyajobi et al., 1999; Pittenger et al., 1999), and the cultures contained mRNA for bone cell specific genes such as osteopontin and osteocalcin, none of which was expressed at detectable levels in freshly isolated AGM cells (Fig. 2B) (Bruder et al., 1997; Oyajobi et al., 1999). Additionally, *Cbfa1* gene expression was upregulated after differentiation into the osteogenic lineage. The osteogenic phenotype of the cultures was further confirmed by the formation of mineralized nodules (Banfi et al., 2000), as revealed by Alizarin Red staining (Fig. 2C). The incidence of colonies that followed osteogenic differentiation, as measured by ALP expression, was 35.6±18.6% of the total colony count. When stimulated with dexamethasone and insulin, AGM cells formed colonies in which the cells acquired a typical adipocyte morphology, displaying abundant refractile intracellular vesicles containing

Table 1. Cell-seeding densities of tissues used for differentiation assays

Embryonic tissue	Osteogenic (cells/cm^2)	Adipogenic (cells/cm^2)	Chondrogenic (cells/pellet)
E11 head, somites, limb buds, heart, liver, and vitelline and umbilical arteries	5×10^3-5×10^4	5×10^3-5×10^4	5×10^5-1×10^6
E11, E12 YS	5×10^3-5×10^4	5×10^3-5×10^4	5×10^5-5×10^6
E11, E12 AGM	1×10^3	5×10^3	1×10^5
E14 liver	1×10^4	1×10^4	1×10^6
Neonatal BM	1×10^5-1.5×10^5	1×10^5-1.5×10^5	1×10^6-2×10^6
Adult BM	1.5×10^5	1.5×10^5	1×10^6
E11, E12, E14, E17 blood	5×10^5	5×10^5	1×10^6-5×10^6

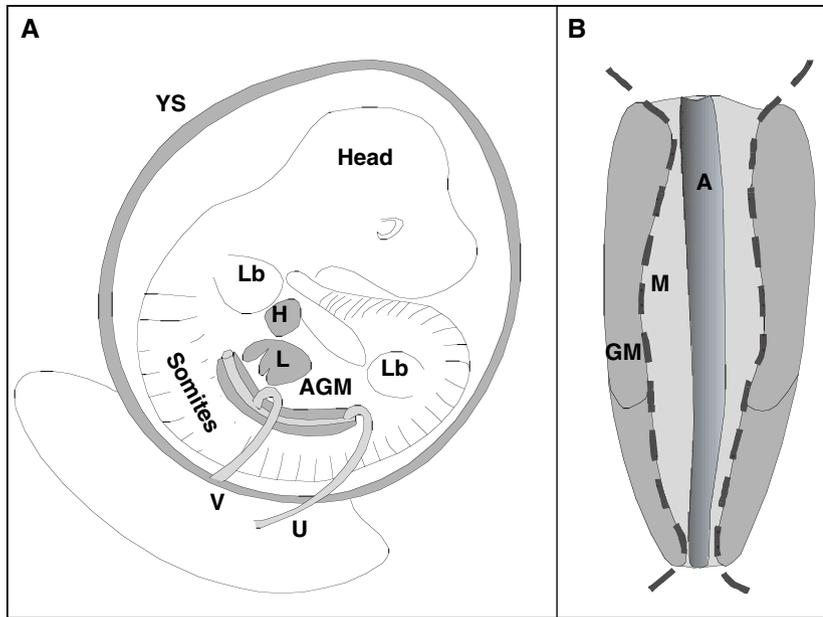YS, yolk sac; AGM, aorta-gonad-mesonephros; BM, bone marrow.

Development

Fig. 1. (A) Schematic drawing of a mouse embryo at E11. Yolk sac (YS), head, somites, limb buds (Lb), heart (H), liver (L), umbilical (U) and vitelline (V) arteries, and the aorta-gonad-mesonephros (AGM) region were dissected and cells isolated for further testing. (B) AGM region, divided into the aorta (A), with the adjacent mesenchyme (M), and the gonads and mesonephros (GM) that constitute the urogenital ridges.

lipids (Fig. 2D), and upregulated the expression of the adipocytic marker peroxisome proliferation-activated receptor γ (*Pparg*) (Pittenger et al., 1999) (Fig. 2B). Under these conditions, the percentage of colonies that followed the adipogenic pathway was 46.0±9.0% of the total colony count. The chondrogenic potential of AGM cells was revealed by the formation of foci. These foci contained rounded chondrocyte-like cells embedded in an abundant extracellular matrix rich in proteoglycans (Fig. 2E,F; Toluidine Blue staining). Both the cells and the extracellular matrix were strongly positive for collagen type II (Fig. 2G). By these cartilage-associated criteria, these results indicate the presence of chondrogenic

Table 2. Mesenchymal differentiation potential of E11 tissues

Embryonic tissue	Differentiation potential		
	Osteogenic	Adipogenic	Chondrogenic
YS	–	–	–
Head	–	–	–
Somites	–	–	+*
Limb buds	–	–	+*
Heart	–	–	–
Liver	–	–	–
Vitelline and umbilical arteries	–	–	–
AGM	+	+	+

Embryonic tissues were dissected and cells were cultured under osteogenic, adipogenic and chondrogenic conditions as described in the Materials and methods.

+, capacity of differentiation into the specific lineage.

–, lack of potential to differentiate into the specific lineage.

*Attributable to the presence of an already differentiated prechondrogenic mass.

From the tissue remnants remaining after the dissection of the YS, head, somites, limb buds, heart, liver, vitelline and umbilical arteries, and the AGM region, we occasionally found some limiting osteogenic and chondrogenic activity. Thusfar, we have not been able to identify the specific cells responsible for the activity.

YS, yolk sac; AGM, aorta-gonad-mesonephros.

progenitors in the AGM (Dennis et al., 1999; Pittenger et al., 1999).

Cells from YS, vitelline and umbilical arteries, head, heart and liver did not contain detectable progenitors for any of the evaluated lineages, even when cultured at high-cell densities. Although somites and limb buds contained cells able to form cartilage in vitro, this is due to the already differentiating prechondrogenic mass that constitutes these tissues (Cheah et al., 1991) and is not attributable to mesenchymal stem/progenitor cells. Thus, mesenchymal stem/progenitor cells are exclusively localized in the AGM region of the E11 embryo.

Sublocation of mesenchymal stem/progenitor cells within the E11 AGM

To more specifically localize mesenchymal stem/progenitor cells within the AGM, the dorsal aorta with its surrounding mesenchyme (AoM) and the urogenital ridges (UGRs) (Fig. 1B) were subdissected and analyzed. Differentiation cultures revealed that progenitors with osteogenic potential, as determined by ALP activity, were present throughout the entire AGM region. In the whole AGM region, osteogenic precursors were present at a frequency of $1.93\pm0.40/10^4$ cells [Fig. 3A; there was no contribution of ALP-positive primordial germ cells in the AGM region to this frequency (see figure legend)]. The UGRs had a slightly higher frequency of these cells $(2.27\pm0.25/10^4$ cells), and the AoM, a 2-fold lower frequency $(1.07\pm0.40/10^4$ cells). The absolute number of osteogenic progenitors was also higher in the UGRs than in the AoM (Table 3). Because the number of osteogenic precursors in the whole AGM region was higher than the sum of these cells in the AoM and UGRs, either a loss of osteogenic progenitor cells is incurred during subdissection, or cells of both subregions are required for optimal differentiation potential to be revealed.

Progenitor cells with the ability to differentiate into the adipocytic lineage were exclusively detected in the UGRs at a frequency of $5.58\pm1.18/10^4$ cells. This represents a 2-fold higher frequency than in the whole AGM $(2.92\pm0.72/10^4$ cells; Fig. 3B). The AoM was devoid of such cells, even when

cultured at high-cell numbers. Thus, in absolute numbers, all adipogenic progenitors localize to the UGRs (Table 3).

Chondrogenic progenitors were present at a lower frequency in the AGM ($0.85\pm0.22/10^4$ cells; Fig. 3C) than either osteogenic or adipogenic progenitors. Such progenitors were located both in the UGRs and AoM at frequencies of $0.70\pm0.18/10^4$ and $0.48\pm0.17/10^4$ cells, respectively. Again, the

AoM appeared to contain fewer progenitors than the UGRs, although this difference was not statistically significant ($P=0.06$).

In summary, cultures from both AoM and UGRs revealed that osteogenic and chondrogenic progenitor cells are distributed throughout the entire AGM region, whereas precursor cells for the adipogenic lineage are exclusively located in the UGRs. Moreover, both the frequency and absolute number of adipogenic progenitors in the UGRs (Table 3) are substantially higher than the frequency and absolute number of progenitors for the other two lineages, indicating that this site provides a particularly favorable microenvironment for the adipogenic differentiation. These findings suggest that at this developmental stage, the mesenchymal progenitors found in the AoM have a more restricted potential than the progenitors detected in the UGRs, or that adipogenic cells arise from a different progenitor than osteogenic and chondrogenic cells.

Mesenchymal stem/progenitor cells progressively increase in number in hematopoietic sites during development

The finding that mesenchymal progenitors are exclusively localized in the E11 AGM region prompted us to examine other hematopoietic sites for such activity. AGM, YS, liver and BM cells were isolated and tested at several developmental stages. At E12 the AGM region continued to harbor mesenchymal progenitors with potential for osteogenic, adipogenic and chondrogenic lineages, while no detectable progenitors were found in the highly hematopoietic E12 YS. Mesenchymal progenitors were present in the E14 liver, neonatal BM and adult BM and were capable of differentiating into all three lineages. Thus, mesenchymal cells, similar to those previously found in adult BM (Kuznetsov et al., 1997; Pereira et al., 1998; Pittenger et al., 1999; Prockop, 1997), are present in most primary hematopoietic tissues throughout development. The frequency and absolute number of osteogenic, adipogenic and chondrogenic progenitors was determined in each of these hematopoietic sites and the results are shown in Fig. 4.

From E11 to E12, the frequency of osteogenic progenitor cells in the AGM region remained approximately constant (Fig. 4A). However, the absolute number of these cells increased (from 81 ± 10 to 102 ± 7; $P=0.04$), suggesting their expansion within this region. Such progenitors were absent or below the detection level in the E11 liver. At E14 the liver contained 0.090 ± 0.020 osteogenic progenitors per 10^4 cells. Despite the fact that the frequency of osteogenic progenitors in the E14 liver was much lower (21-fold) than in the E11/12 AGM, their absolute number was significantly higher: 173 ± 31 cells in the E14 liver as compared to 102 ± 7 cells in the E12 AGM ($P=0.008$). This result suggests that progenitors for the osteogenic lineage are amplified in the liver. In neonatal and adult BM, the frequency of osteogenic progenitors was also significantly lower ($0.045\pm0.013/10^4$ cells and $0.037\pm0.008/10^4$ cells, respectively; $P<0.001$) than in the E14 liver. With regard to the absolute number of these cells in neonatal and adult BM, we estimated 439 ± 121 osteogenic precursors at the neonatal stage and 1100 ± 244 in the adult. This 2.5-fold
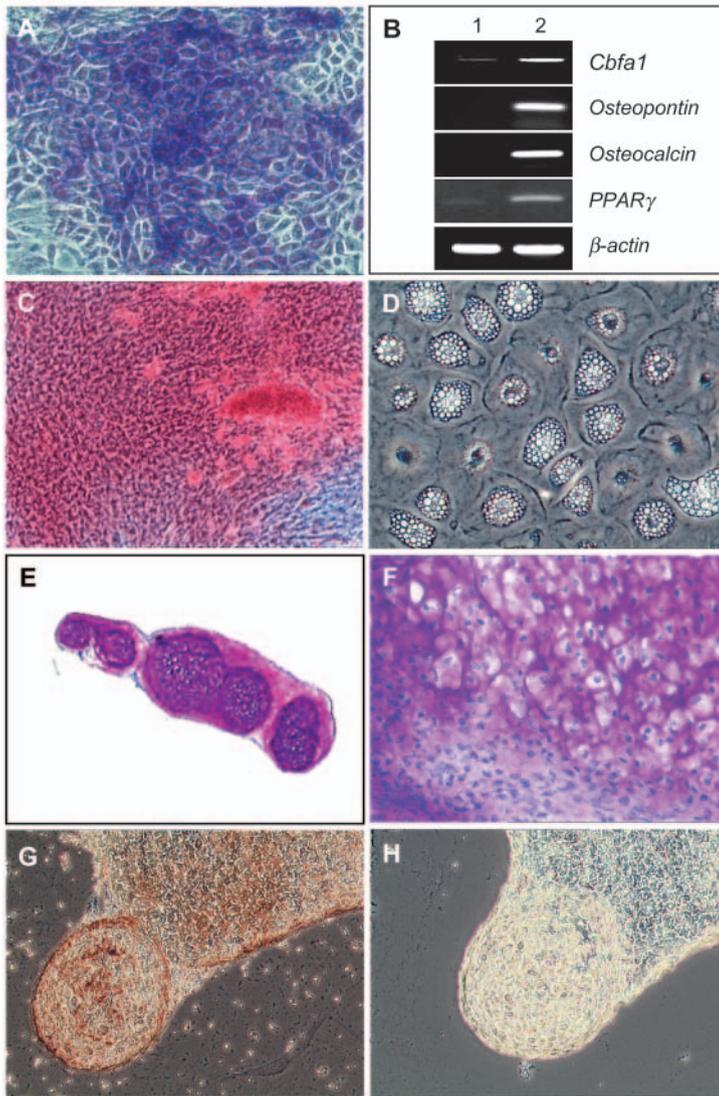

Fig. 2. Differentiation potential of AGM cells. (A-C) Osteogenic cell differentiation. After 10 days in osteogenic permissive medium, cell colonies stained positive for ALP activity (A), and cultures expressed the osteoblastic markers osteopontin and osteocalcin (B, lane 2). Neither marker was expressed in freshly isolated AGM cells (B, lane1). Differentiated cultures also showed an upregulation of the osteogenic marker *Cbfa1* (B, lane 2). At day 21, mineralization of the cultures was observed with Alizarin Red (C). (D) Adipogenic differentiation and colony formation. After stimulation with adipogenic-inductive medium for one week, *Pparg* was upregulated (B, lane 2) when compared with the expression of this marker in freshly isolated AGM cells (B, lane 1). (E-H) Chondrogenic differentiation. After 21 days in chondrogenic medium, cells formed several cartilaginous foci (E) with an extracellular matrix rich in proteoglycans, as detected by Toluidine Blue staining (F). Foci were also strongly positive for collagen type II, as shown by the brown DAB staining (G). A secondary antibody control is shown in H.

Fig. 3. Frequency of (A) osteogenic, (B) adipogenic and (C) chondrogenic progenitors in E11 AGM and its subregions, the dorsal aorta with its surrounding mesenchyme (AoM) and the urogenital ridges (UGRs). Osteogenic and adipogenic progenitor frequencies were determined based on the number of colonies displaying ALP activity and refractile, lipid-containing vesicles, respectively. Chondrogenic progenitor frequency was determined based on the number of cartilagenous foci after 21 days of aggregate culture. Data are expressed as mean±s.d. (*n*=3 to five independent experiments). Because the AGM region also contains primordial germ cells expressing ALP, the specificity of this marker with regard to osteogenic commitment was addressed. Oct4-GFP transgenic AGM cells were sorted and evaluated in osteogenic cultures for ALP-expressing colony frequency. The frequency of osteogenic progenitors in the primordial germ cell depleted (sorted Oct4-GFP negative) fraction was identical to unsorted AGM cells ($1.32\pm0.41/10^4$ vs $1.30\pm0.14/10^4$, respectively). Thus, primordial germ cells are not contributing to the ALP-expressing colony frequency after culture in osteogenic-inductive conditions.
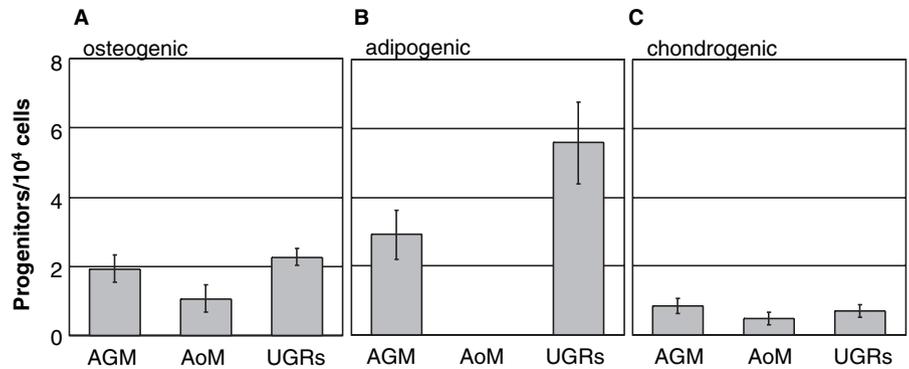
increase in mesenchymal stem/progenitor cell number indicates that expansion is also occurring within the BM microenvironment.

Like the osteogenic progenitor frequency, the frequency of adipogenic progenitors in the AGM did not vary significantly from E11 to E12 (Fig. 4B). Nonetheless, the absolute number of these cells per AGM did increase slightly (*P*=0.1). In the liver, these progenitors were detected only beginning at E14 and, although their frequency in this tissue was very low when compared with the E11/12 AGM, the absolute number of adipogenic progenitors per tissue was higher (244 in E14 liver as compared with 124-183 in the E11/12 AGM). Both in neonatal and adult BM, the frequency of adipogenic progenitors decreased more than 10-fold when compared with the progenitor frequency in the E14 liver. The absolute number of these progenitors decreased in the neonatal BM to 72±25, whereas this number increased to 191±53 in the adult stage. Thus, throughout development the number of adipogenic progenitors is variable, between 100-250 progenitors per tissue.

The frequency of chondrogenic progenitors in the AGM region did not statistically vary from E11 to E12, but a developmentally progressive decrease in the frequency was observed in the E14 liver (*P*=0.01, when compared with E11/12 AGM), and in neonatal and adult BM (*P*<0.001, when compared with E14 liver). However, a significant (*P*=0.007) increase in the absolute number of chondrogenic progenitors

was observed in E14 liver (143±37) when compared with E12 AGM (34±7). This increased number of progenitors persisted in the adult BM (Fig. 4C).

In summary, spatial quantification of mesenchymal progenitors during development demonstrates that although progenitor frequency decreases, the absolute numbers of progenitors increases to a plateau level. Osteogenic progenitors predominate in adult BM, while adipogenic progenitors predominate in the AGM. Taken together, these results suggest a developmentally progressive amplification of mesenchymal progenitors in each of the hematopoietic sites, AGM, liver and BM.

Mesenchymal progenitors are present in embryonic blood

Although there is a progressive appearance and increase in mesenchymal progenitor activity in the liver and BM during development, it is undetermined whether these tissues are seeded through the circulation with progenitors arising from, for example, the AGM region. It is generally accepted that for the hematopoietic system, hematopoietic cells (erythroblasts) arising in the YS, migrate and colonize the liver rudiment at late E9 (Houssaint, 1981; Johnson and Jones, 1973). To investigate the possibility that migration of mesenchymal progenitors may occur between tissues, embryonic blood was tested for the presence of such cells at E11, E12, E14 and E17. At E11 and E17, even with high cell seeding densities, no progenitors for any of the three mesenchymal lineages could be detected in circulating blood. However, both at E12 and E14, stem/progenitor cells for the osteogenic, adipogenic and chondrogenic lineages were detected in circulating blood (Fig. 5). The frequency of these cells in blood both at E12 and E14 was extremely low (on average 30-500 times lower than progenitor frequency in E12 AGM and E14 liver). These results suggest that at least some mesenchymal stem/progenitor cells may relocate to the different hematopoietic sites through the circulation.

Is the presence of mesenchymal progenitors dependent upon HSC activity?

To evaluate whether the location of mesenchymal progenitors

Table 3. Sub-localization of mesenchymal progenitors in the E11 AGM

| | Differentiation potential | | |
	Osteogenic	Adipogenic	Chondrogenic
AGM	81±10	124±32	28±1
AoM	18±4	0±0	8±4
UGR	31±7	96±22	12±4

Absolute number of osteogenic, adipogenic and chondrogenic progenitors per AGM or subdissected tissue.

AGM, aorta-gonad-mesonephros; AoM, aorta plus the surrounding mesenchyme; UGR, urogenital ridges.

The results are expressed as mean±s.d. (*n*=3 to five different experiments).

in hematopoietic sites is associated with hematopoietic activity, studies were performed using embryos deficient in HSC activity. The Runx1 transcription factor is required for the generation of HSCs in the AGM region, and this block in HSC

<div style="writing-mode: vertical">Development</div>

A

B

C

Fig. 4. Frequency and absolute number of (A) osteogenic, (B) adipogenic and (C) chondrogenic progenitors in hematopoietic sites through development. Osteogenic and adipogenic progenitor frequencies and numbers were determined based on the number of colonies displaying the appropriate phenotype. Chondrogenic progenitor frequency and number was determined based on the number of cartilage foci after 21 days of aggregate culture. Data are expressed as mean±s.d. (*n*=3 to six independent experiments). Absolute number determinations in the neonatal and adult BM used the following values: newborn BM, 9.8×10^7 cells; adult BM, 30.0×10^7 cells (Harrison, 1993). These values were extrapolated from those of Stewart et al. (Stewart et al., 1993).

development in Runx1$^{-/-}$ embryos results in fetal liver anemia and early lethality at E12.5 (Cai et al., 2000; North et al., 1999). We used E11 Runx1$^{-/-}$ AGMs to assess whether the presence and frequency of mesenchymal progenitors was affected by the absence of HSCs. As shown in Table 4, despite the severe hematopoietic defect, the AGM region of Runx1$^{-/-}$ embryos harbors osteogenic, adipogenic and chondrogenic progenitors in frequencies identical to those found in wild-type littermates. These data imply that, at this developmental stage, the presence and frequency of mesenchymal progenitors in hematopoietic sites is independent of HSC emergence and/or function.

Discussion

To better understand the development of mesenchymal cell lineages, particularly within the hematopoietic microenvironment, our work focused on the mapping, frequency and differentiation potential of mesenchymal progenitors at several ontogenic stages in the mouse. We found that mesenchymal progenitors within the embryo are exclusively localized at E11 within the hematopoietic tissue that is the first source of adult-type HSCs, the AGM region. The AGM is a potent microenvironment for several stem cell types, such as HSCs and primordial germ cells. Hence, it is not surprising that mesenchymal stem/progenitor cells are also localized there. Indeed, Minasi and colleagues have found mesodermal progenitors (mesangioblasts) within the E9 aorta with differentiation potential for all mesodermal hematopoietic and non-hematopoietic lineages (Minasi et al., 2002). The fact that mesangioblasts isolated from the aorta possess a greater differentiation potential (hematopoietic, endothelial, myogenic, osteogenic and chondrogenic) and are found earlier than our AGM mesenchymal progenitors suggests either that we are examining a distinct cell type or that the E9 mesangioblasts are the precursors to the E11 mesenchymal progenitors.

Rather than being localized in the aorta with mesangioblasts, we found mesenchymal stem/progenitor cells for osteogenic, adipogenic and chondrogenic lineages localized to the UGRs. We also found mesenchymal progenitor cells localized to the aorta, but these had a more restricted potential (osteogenic and chondrogenic). AGM subregional localization differences are also known for HSCs. At E11, HSCs localize to the aorta, but by E12, they can be found both in the aorta and UGRs (de Bruijn et al., 2000). Moreover, other investigators have demonstrated that cells readily migrate from the mesonephros to the gonads at E10/E11 (Martineau et al., 1997; Perez-Aparicio et al., 1998). Thus, as development proceeds during the crucial period from E9 to E11, there appears to be a widespread and ongoing spatial reorganization and/or maturation/differentiation of several stem cells and progenitors in the AGM region, and this most likely includes mesenchymal stem/progenitor cells.

We have shown that at E12 the AGM continues to harbor mesenchymal stem/progenitor cells, and, thereafter, we found such cells in the E14 liver, and later in the neonatal and adult BM. In all these locations, stem/progenitor cells possessed similar potentials, being able to differentiate into cells of the osteogenic, adipogenic and chondrogenic lineages. Their frequency sequentially decreased from site to site during development. However, the absolute number of mesenchymal
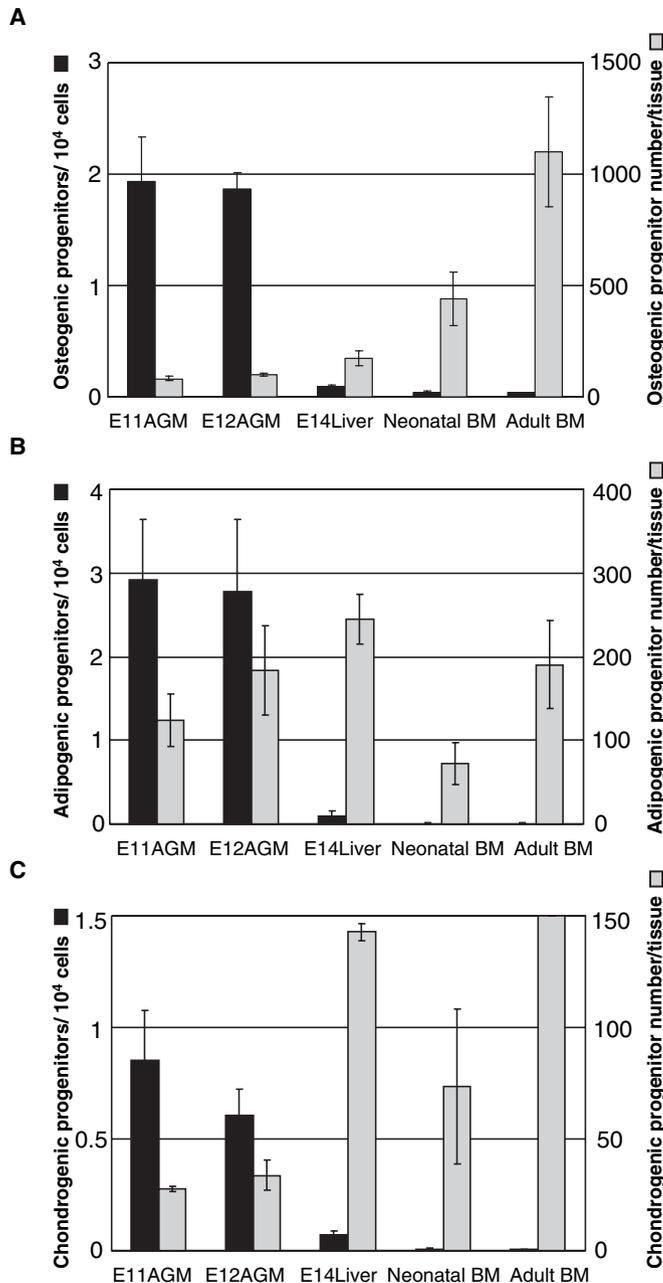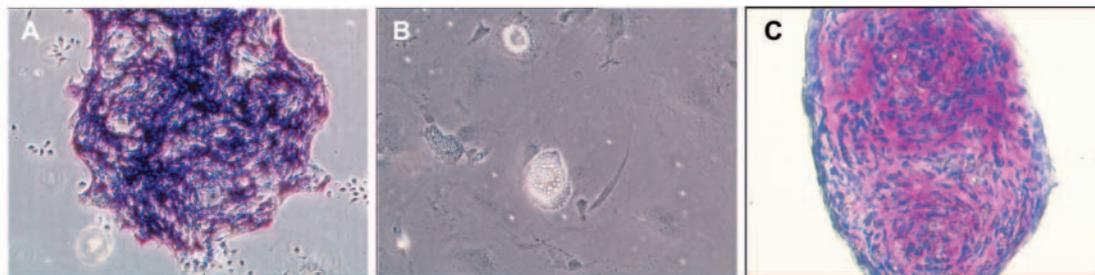
Fig. 5. Presence of stem/progenitor cells in cultures of E12 blood. (A) Osteogenic cell differentiation after 10 days of treatment in osteogenic permissive medium. Colonies stained positive for ALP. (B) Adipogenic differentiation after stimulation with adipogenic-inductive medium for 10 days; adipocytes are shown. (C) Chondrogenic differentiation after 21 days in chondrogenic medium. E12 blood cells formed a small cartilage-like pellet, in which the extracellular matrix was rich in proteoglycans, as detected by Toluidine Blue staining.

Table 4. Frequency of mesenchymal progenitors in the E11 AGM of Runx1 mutant embryos

	Differentiation potential		
	Osteogenic	Adipogenic	Chondrogenic
Runx1$^{-/-}$	1.20±0.45	3.30±0.71	0.6
Runx1$^{+/+}$	1.83±0.98	2.80±1.13	0.6

Results are expressed as mean±s.d./10^4 AGM cells.

Due to the difficulty in attaining Runx1$^{-/-}$ embryos only one experiment was performed, and in this experiment osteogenic and adipogenic differentiation and frequency analysis were done in triplicate; for the chondrogenic experiments only one sample per group was used.

stem/progenitor cells in these tissues increased with developmental time to reach relative plateau levels. Hence, our results demonstrate that AGM, liver and BM are excellent environments for the maintenance and expansion of mesenchymal stem/progenitor cells. Although, the increases in mesenchymal stem/progenitor cells parallel the increase in HSC numbers in these tissues throughout ontogeny (Kumaravelu et al., 2002; Morrison et al., 1995), a possible developmental relationship between mesenchymal and hematopoietic cells leading to a parallel and coordinate development of these systems still needs to be proven.

As shown in Table 1, the plating densities at which mesenchymal progenitors could be detected increased from site to site during ontogeny, implicating a decrease in the frequency of detectable mesenchymal progenitors throughout development. This decrease reflects either a 'real' decrease in the incidence of mesenchymal progenitors, or it could be related to the cells constituting the microenvironment that would differentially affect the in vitro proliferation and differentiation capacity of the progenitor cells. Because we could detect an increase in the absolute numbers of these progenitors from hematopoietic site to hematopoietic site during development, the microenvironments of these organs seem to be supportive of mesenchymal progenitor expansion and differentiation, and, therefore, the decrease in frequency is most likely due to the large increase in cells of other lineages in these tissue rudiments.

Thus, we have shown that during development: (1) mesenchymal stem/progenitor cells are present in the major hematopoietic sites; (2) hematopoietic organs provide a good microenvironment for the maintenance and expansion of these

cells; and (3) a small part of the mesenchymal progenitor population is in the circulation. These findings provide new information supporting the notion that the origins and formation of the hematopoietic supportive microenvironment and the hematopoietic system closely parallel each other.

It should be mentioned that our studies were not carried out at a clonal level and thus it is difficult to discern between true MSCs and mesenchymal progenitors within the evaluated population. Nevertheless, because in each evaluated site the frequency and number of precursor cells varied from lineage to lineage, it is likely that the analyzed cell population actually consists of both true stem cells and progenitors at several stages of differentiation.

Mesenchymal-derived stromal cells are known to be essential in regulating the balance between HSC self-renewal and differentiation from the earliest stages in the AGM to the adult BM. Stromal cell lines with hematopoietic supportive ability have been isolated from the AGM region and its associated subregions (Oostendorp et al., 2002a; Oostendorp et al., 2002b), and indeed, one of the most supportive stromal clones is derived from the UGRs. Highly supportive stroma cells have also been isolated from the fetal liver (Moore et al., 1997) and the adult BM. Moreover, these stromal clones, including AGM-derived clones, have the potential to differentiate to most of the mesenchymal lineages in appropriate culture conditions (C. Durand, E. Haak and E.D., unpublished). This, taken together with the fact that mesenchymal stem cells from BM enhance engraftment of HSCs after transplantation (Almeida-Porada et al., 2000), suggests that both HSCs and mesenchymal-derived cells work in concert with each other.

While it is clear that HSCs require the presence of stromal cells for their maintenance and differentiation, it was unclear whether the reverse was true. Our results in Runx1 deficient embryos, demonstrate that the presence, differentiation potential and frequency of mesenchymal progenitor/stem cells in E11 AGM is not dependent on HSC activity. Although Runx1 deficiency leads to the arrest of HSC emergence and/or function, it is uncertain whether this transcription factor also acts within mesenchymal lineage cells to affect HSC support. We have been able to isolate hematopoietic supportive stromal cells from Runx1$^{-/-}$ E11 AGMs, strongly supporting the notion that the Runx1 transcription factor does not play a crucial role in the generation of this mesenchymal stem/progenitor cell compartment (E. Haak and K. Harvey, unpublished).

In contrast to our findings that mesenchymal stem/progenitor cells are localized to the AGM and other major (highly vascularized) hematopoietic tissues throughout development, we found no mesenchymal stem/progenitor cells in the highly vascularized midgestation YS. This may not be surprising as the YS is a simple two-layered structure and it becomes extinct in late gestation. We also found site-specific variations in differentiation potential in the other major hematopoietic sites. For example, whereas osteogenic and chondrogenic progenitors were both located in the AoM and UGR sub regions, adipogenic progenitors were only present in the UGR part of the AGM. Thus, it appears that specific tissues limit differentiation potential, influence the mesenchymal lineage hierarchy and/or are unable to maintain mesenchymal stem/progenitor cells. Some studies examining the mesenchymal differentiation hierarchy have shown that the adipogenic lineage diverges and becomes independent earlier than the osteogenic and chondrogenic lineages (Banfi et al., 2000). Hence, future studies should focus on the mesenchymal lineage hierarchy in the different ontogenic sites to determine the mechanisms by which mesenchymal differentiation occurs.

In conclusion, mesenchymal stem/progenitor cells were detected in the major hematopoietic sites during mouse development: in the AGM and liver at midgestational stages, and later in neonatal and adult BM. These cells are able to amplify within the hematopoietic sites. At E11, the Runx1 HSC deficiency does not affect the presence, differentiation potential or frequency of mesenchymal progenitors. As mesenchymal stem/progenitor cells are found in the midgestation circulation, lineage tracing through development will be our future focus, so as to determine whether indeed, mesenchymal stem/progenitor cells migrate and colonize the major hematopoietic tissues to supply the hematopoietic supportive microenvironment.

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