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Hematopoietic Stem Cells Localize to the Endothelial Cell Layer in the Midgestation Mouse Aorta

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Summary

The emergence of the first adult hematopoietic stem cells (HSCs) during mammalian ontogeny has been under intense investigation. It is as yet unresolved whether these first HSCs are derived from intraembryonic hemangioblasts, hemogenic endothelial cells, or other progenitors. Thus, to examine the spatial generation of functional HSCs within the mouse embryo, we used the well-known HSC marker, Sca-1, and a transgenic approach with an *Ly-6A* (Sca-1) *GFP* marker gene. Our results show that this transgene marker is expressed in all functional HSCs in the midgestation aorta. Immunohistology of aorta-gonads-mesonephros (AGM) regions show that GFP$^+$ cells are specifically localized to the endothelial layer lining the wall of the dorsal aorta but not to the mesenchyme, strongly suggesting that HSC activity arises within a few cells within the endothelium of the major vasculature.

Introduction

During adult stages, the vertebrate hematopoietic system is constantly renewed from hematopoietic stem cells (HSCs) harbored in the bone marrow. These HSCs are characterized by functional repopulation properties elaborated after transplantation into adult recipients depleted for endogenous hematopoietic activity. The defining characteristics of HSCs are long-term, high level repopulation of all hematopoietic lineages and the ability to self-renew (Lemischka, 1991; Spangrude et al., 1991). During ontogeny, the first adult-type HSCs arise in the aorta-gonads-mesonephros (AGM) region, as shown by direct transplantation of AGM cells into adult recipients (Muller et al., 1994) or by transplantation of such cells after AGM explant culture (Medvinsky and Dzierzak, 1996). Further investigation of HSC emergence within the embryo has demonstrated their presence in the regions of major vasculature: the vitelline and umbilical arteries and the dorsal aorta (de Bruijn et al., 2000). Furthermore, within all mammalian and nonmammalian vertebrates analyzed, clusters of hematopoietic cells have been observed along the vitelline and umbilical arteries as well as the ventral wall of the dorsal aorta (Garcia-Porrero et al., 1995; Shalaby et al., 1997; Tavian et al., 1999; Wood et al., 1997), suggesting that hematopoietic cell emergence occurs in close association with the major vasculature of the embryo.

Indeed, many years ago, based on microscopic observations of yolk sac blood islands, it had been proposed that there exists a common mesodermal precursor cell, the hemangioblast, for hematopoietic and endothelial lineages (Murray, 1932). Gene targeting experiments have yielded some insight into the relationship between these lineages and have shown that the Flk-1 receptor tyrosine kinase, for example, is required for development of both the endothelial lineage and the hematopoietic lineage (Shalaby et al., 1997). However, unlike the coordinated emergence of hematopoietic and endothelial cells in the yolk sac, the dorsal aorta is formed before the emergence of adult repopulating HSCs, suggesting a slightly different type precursor or hemangioblast. Immunohistochemical analyses of the AGM region reveal overlap in the expression of hematopoietic and endothelial markers in the clusters of cells that appear to be emerging from the ventral wall of the dorsal aorta (Marshall and Thrasher, 2001). The most interesting expression pattern is exhibited by the Runx1 (previously known as Cbfa2 or AML1) transcription factor (North et al., 1999), which has been shown to be required for HSC activity in the AGM but does not appear to affect the major vasculature or early yolk sac hematopoiesis (Cai et al., 2000; Wang et al., 1996; Okuda et al., 1996; Mukouyama et al., 2000). At embryonic day 10 (E10), *Runx1* expression is found in the hematopoietic clusters and endothelial cells lining the walls of the vitelline and umbilical arteries and the ventral wall of the dorsal aorta (North et al., 1999). Additionally, expression is found in some mesenchymal cells underlying the ventral endothelial cells of the dorsal aorta. Taken together, these studies suggest that adult repopulating HSCs are derived from precursors within the hematopoietic clusters, endothelium, and/or underlying mesenchyme, and only the use of further markers will yield a precise identification of the HSC precursor. Recently, flow cytometric sorting of Runx1 positive cells together with combinations of antibodies recognizing other marker proteins on hematopoietic, endothelial, and mesenchymal cells has supported a mesenchymal and/or endothelial origin for HSCs (North et al., 2002 [this issue of *Immunity*]).

For many years, the Sca-1, GPI-linked cell surface glycoprotein has been used as a faithful marker of murine HSCs from various sources; i.e., adult bone marrow, fetal liver, and yolk sac (Spangrude et al., 1988; Huang and Auerbach, 1993). Sorting on the basis of the monoclonal antibody E13-161.7 specific for the Sca-1 epitope yields about a 100-fold enrichment of HSCs from adult bone marrow (Okada et al., 1992; Spangrude et al., 1988). Sca-1 is encoded by the strain specific allelic genes, *Ly-6E* and *Ly-6A* (Khan et al., 1990; Sinclair and Dzierzak, 1993; Stanford et al., 1992; van de Rijn et al.,

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1989), which are members of the large *Ly-6* gene family (Kamiura et al., 1992; LeClair et al., 1986). Sca-1 protein expression is complex within *Ly-6A* and *Ly-6E* strains of mice (Kimura et al., 1984; Spangrude and Brooks, 1993; van de Rijn et al., 1989). *Ly-6A* strains of mice express Sca-1 on virtually all (99%) of marrow-repopulating cells from the adult bone marrow, while *Ly-6E* strains express Sca-1 on only 25% of these cells (Spangrude and Brooks, 1993). Despite the allelic differences, the Sca-1 marker should be useful in identifying and localizing the first HSCs as they emerge in the AGM region. The relatively small size of the *Ly-6A/E* gene locus (14 kb) which contains all the transcriptional regulatory elements of these alleles (Khan et al., 1990, 1993; McGrew and Rock, 1991; Sinclair and Dzierzak, 1993; Ma et al., 2001; Sinclair et al., 1996) has been useful in directing *lacZ* marker gene expression in bone marrow HSCs in transgenic mice (Miles et al., 1997; Ma et al., 2002).

Thus, to identify the lineage of cells from which the first functional HSCs emerge in the AGM region, we performed studies using the Sca-1 surface glycoprotein marker and the enhanced green fluorescent protein (GFP) marker within the context of an *Ly-6A* transgene in midgestation mouse embryos. We report here that while only some AGM HSCs express the Sca-1 protein, all AGM HSCs are positive for expression of GFP from the *Ly-6A GFP* transgene. Immunohistological analyses reveal expression of the GFP marker within a single layer of cells lining the wall of the dorsal aorta and the vitelline/umbilical arteries. Together with functional data demonstrating the presence of HSC activity exclusively in the GFP positive fraction of the aorta region, our results strongly suggest that the first HSCs within the midgestation embryo are localized within the endothelial cell layer lining the wall of the dorsal aorta.

Results

The Sca-1 Antigen Is Expressed on Some AGM HSCs

To examine whether the Sca-1 antigen is expressed on AGM HSCs, we sorted Sca-1$^+$ cells from E11 AGM cell suspensions and performed transplantation experiments to test for functional hematopoietic repopulation of irradiated adult recipient mice by the donor cells. A representative fluorescence profile (Figure 1A) shows that 3.8% (range 1.95%–3.9%) of E11 AGM cells are Sca-1$^+$. At greater than 4 months posttransplantation, recipients were tested for the presence of donor cells by PCR of peripheral blood DNA (donor cell marker is a *lacZ* or human β-*globin* transgene). When transplanted at 1 embryo equivalent (ee) of sorted cells per recipient, adult repopulating HSCs were found in both the Sca-1$^+$ and Sca-1$^-$ fractions (Figure 1B) (donor engraftment ranging from 15% to 38%). This result may reflect a low or negative expression of Sca-1 by some HSCs as they emerge in the E11 AGM.

Previously, we had shown that all E11 AGM HSCs are c-kit$^+$ (Sanchez et al. 1996). To further subfractionate AGM HSCs, we sorted E11 AGM cells on the basis of c-kit and Sca-1 antigen expression. In these experiments, we confirm that all functional adult repopulating HSCs are c-kit$^+$ and that they are divided into a Sca-1$^+$ and a Sca-1$^-$ population (data not shown). Thus, the c-kit marker can provide a further enrichment for E11 AGM HSCs.

The Ly-6A GFP Transgene Marks All Functional HSCs from the E11 AGM Region

To study HSC generation and localization within the midgestation mouse embryo, we generated transgenic mice expressing the *GFP* reporter gene under the control of the *Ly-6A* gene regulatory sequences. The enhanced *GFP* gene was inserted into the first untranslated exon of the *Ly-6A* gene (Figure 1C) and was flanked by 4 kb of upstream and 10 kb of downstream genomic sequences containing regulatory elements shown to be necessary for high level specific expression in transgenic mice (Ma et al., 2002).

Since all adult repopulating bone marrow HSCs were found to express the *Ly-6A GFP* transgene, yielding up to a 100-fold enrichment of HSCs (data not shown), we explored whether transgene expression could be used to enrich for HSCs from the AGM region. Sorting experiments were performed on *Ly-6A GFP* E11 AGM cells. Approximately 1.7% (range 1.5%–2.0%) of AGM cells are GFP$^+$ (Figure 1D). Sorted GFP$^+$ and GFP$^-$ cells were transplanted at cell doses of 2, 1, and 0.3 ee (average of 3×10^3 GFP$^+$ cells/ee). At greater than 4 months posttransplantation, >10% donor cell engraftment (ranging from 15% to 70%) was found in recipients receiving either 1 or 2 ee of GFP$^+$ cells (Figure 1E). No donor-derived repopulation was found with 0.3 ee of GFP$^+$ cells or any dose of GFP$^-$ cells. Thus, unlike the Sca-1 marker, *Ly-6A GFP* transgene expression marks all HSCs in the AGM region.

All E11 AGM HSCs Are Localized to the GFP$^+$ Population in the Aorta Region

While previous E11 AGM subdissection studies demonstrated that HSCs are present in the region of the aorta with its surrounding mesenchyme (de Bruijn et al., 2000), we tested whether such cells could be further enriched based on GFP expression. We performed flow cytometric sorting and transplantation of GFP$^+$ cells from the aorta-mesenchyme subregion of E11 *Ly-6A GFP* embryos. The aorta-mesenchyme contained on average 1.9% (1.4% to 3.0% GFP$^+$ cells; see Figure 2A, left panel). Long-term adult repopulating HSC activity (Figure 2A, right panel) was found in the GFP$^+$ fraction of the aorta-mesenchyme when 1 or 2 ee of GFP$^+$ cells was transplanted (average of 1.6×10^3 GFP$^+$ cells/aorta-mesenchyme). No donor cell engraftment was found when the GFP$^-$ fraction was transplanted. Engraftment of recipients, as measured by peripheral blood DNA PCR for the donor cell *GFP* marker at greater than 4 months posttransplantation, ranged from 11% to 100% (data not shown).

To determine whether donor cell hematopoietic engraftment was multilineage, flow cytometric analysis was performed on peripheral blood cells. Representative data of one recipient at 2 months posttransplantation is shown in Figure 2C (bottom panels). GFP expression was found in B220$^+$, CD4$^+$/8$^+$, and Mac-1$^+$ fractions, thus revealing the donor derivation of 35% of the monocytes, 18% of the B cells, and 5.3% of the T

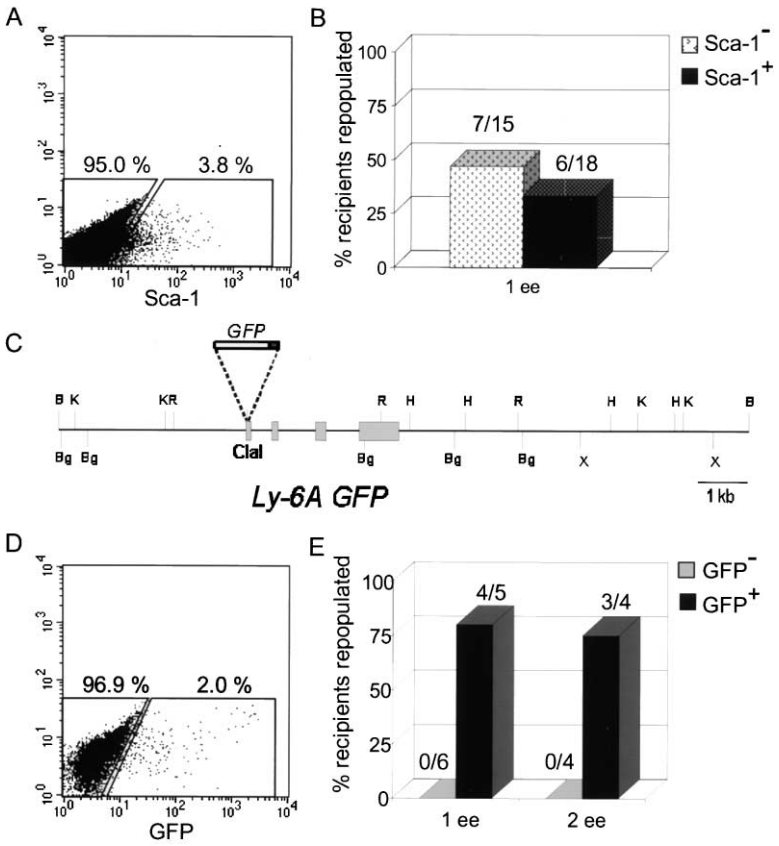

Figure 1. Characterization of E11 AGM Adult Repopulating HSCs for Expression of the Sca-1 Antigen or *Ly-6A GFP* Transgene

(A) E11 AGM region cells were stained with the Sca-1 PE antibody and sorted into Sca-1^+ and Sca-1^- fractions. Percentages in each fraction are indicated.

(B) One embryo equivalent of each fraction of Sca-1^+ (1.0×10^4) and Sca-1^- (25.3×10^4) cells was injected into adult recipient mice, and after 4 months peripheral blood DNA was examined for the presence of donor cells. The number of repopulated animals/total number of animals transplanted is indicated above each column (percentage of recipients repopulated is indicated on *y* axis). Only those animals with >10% donor cells are considered positive. Engraftment of recipients by donor-derived cells as measured by peripheral blood DNA PCR ranged from 15%–38% for Sca-1^+ sorted cells and 16%–36% for Sca-1^- sorted cells. Results are from four independent experiments.

(C) *Ly-6A GFP* transgene construct. The 14 kb BamHI fragment of the *Ly-6A* gene and the insertion of the enhanced *GFP* gene into the Cla1 cloning site is shown. Restriction sites are indicated. B, BamH1; Bg, BglII; K, Kpn1; R, EcoR1; H, HindIII; X, Xba1.

(D) Flow cytometric profile for E11 *Ly-6A GFP* transgenic AGM cells, with percentages of cells within the sorting gates indicated.

(E) Long-term repopulation of recipients (>4 months posttransplantation) receiving 1 or 2 ee of either GFP$^+$ or GFP$^-$ sorted E11 AGM cells. One ee of sorted E11 GFP$^+$ AGM cells is on average 3×10^3 cells, and one ee of GFP$^-$ cells is 17.0×10^4. The number of repopulated animals/total number of animals transplanted is indicated above each column (*y* axis shows percentage of recipients repopulated). Only those recipients with greater than 10% donor positive cells are considered positive. Engraftment ranged from 15%–70% as measured by peripheral blood DNA PCR for the donor cell GFP marker. Results are from four independent experiments.

cells as compared to the control transgenic (top panels). Furthermore, at greater than 4 months posttransplantation, donor marker DNA PCR analysis on two other recipients transplanted with GFP$^+$ aorta-mesenchyme cells revealed repopulation in all hematopoietic tissues and lineages tested (Figure 2D). Engraftment ranged from 21% to 100%. Thus, all long-term, multilineage adult repopulating HSCs from the E11 aorta-mesenchyme can be isolated and enriched within the GFP$^+$ fraction.

The Temporal and Spatial Expression Pattern of Ly-6A GFP Is Correlated with Sites of HSC Generation

The general expression pattern of the *Ly-6A GFP* transgene could be visualized with fluorescence and confocal microscopy. When marker transgene expression was examined in the late E11 AGM region, we found highly fluorescent GFP$^+$ cells in the mesonephric tubules and the Wolffian/Mullerian ducts (Figures 3A and 3B), as we had found previously for the *Ly-6A lacZ* transgene (Ma et al., 2002). However, in contrast to *Ly-6A lacZ* transgenic embryos, we also found punctate GFP expression in the cells along the wall of the dorsal aorta (Figures 3A and 3C). Confocal microscopy revealed the GFP-expressing cells to be dispersed along the circumference and length of the E11 dorsal aorta (Figures 3A and 3C). Compared to the mesonephric tubules in the urogenital ridges (UGR) (Figure 3D), GFP fluorescence of dorsal aorta cells appeared to be of lower intensity (Figure 3C). However, as revealed in transverse sections through the E11 (43 somite pairs [sp]) dorsal aorta (Figure 3F), only a single layer of cells interspersed along the circumference of the aorta was found to be GFP$^+$ (Figure 3E). No GFP expression was found in the mesenchymal tissue directly surrounding the dorsal aorta. GFP expression in the dorsal aorta was found beginning at E9 (24 sp stage) and is confined to only a very few cells in the ventral wall (data not shown). Expression in the aorta becomes more widespread, first along the ventral and then along the dorsal wall at E10 and E11, respectively, and continues until at least E14. In contrast to the dorsal aorta, no GFP expression was observed in the whole yolk sac at E9 or E10 (34 sp stage; Figures 3G and 3H). However, at late E11 (48 sp stage; Figures 3I and 3J) GFP$^+$ cells could be found in the yolk sac but only on very few of the large vessels. Expression in the yolk sac vessels continues at least until E14. Most interestingly, high level GFP expression was found in both the vitelline (Figures 3K and 3L) and umbilical arteries (data not shown) at E10 (38 sp stage). GFP expression in these vessels begins at E9 (24 sp stage) and continues at least until E14 (Figures 3M and 3N). Of the vitelline vessels, high level GFP expression appears to be confined to the artery.

Other sites in the midgestational *Ly-6A GFP* embryo

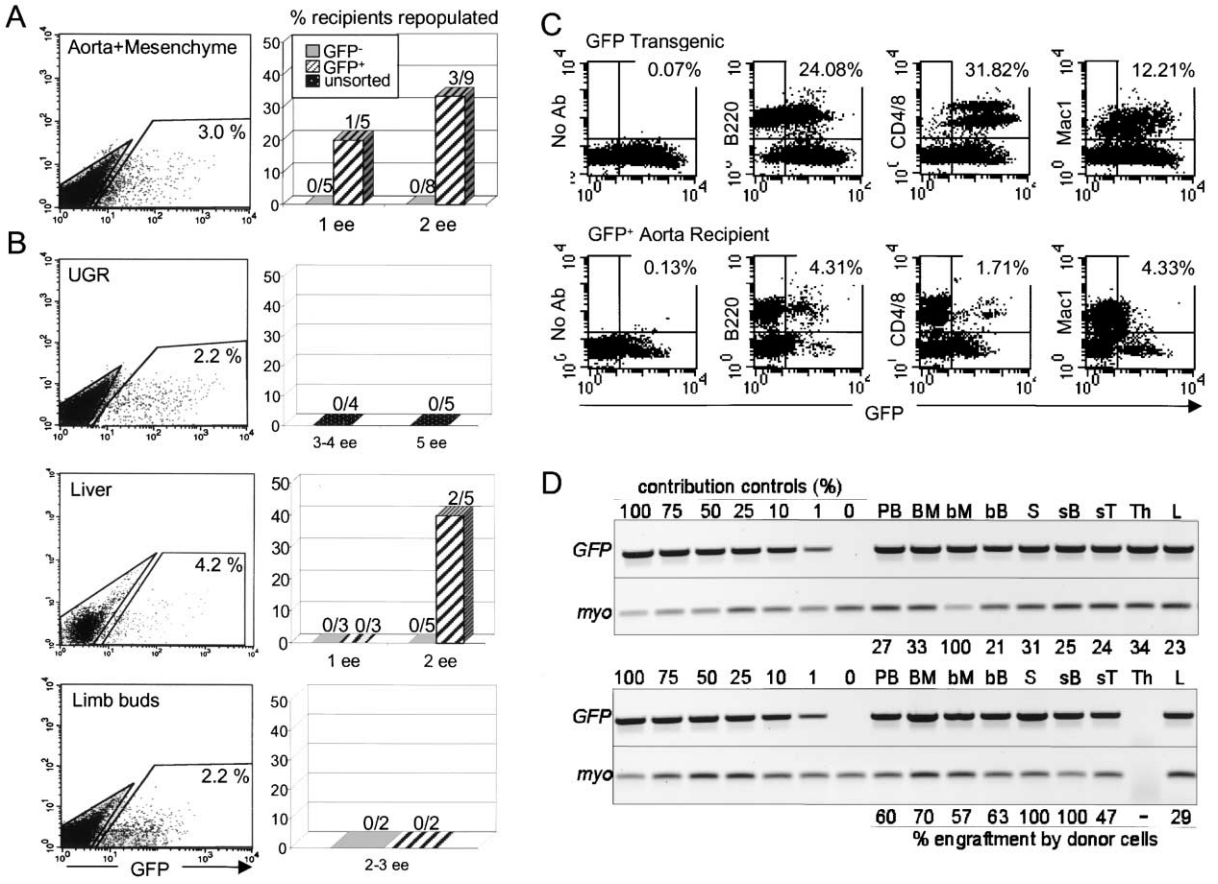

Figure 2. Long-Term Adult Repopulation by GFP-Sorted Cells from E11 Tissues

(A and B) In the left panels flow cytometric profiles and sorting gates are shown for (A) E11 aorta-mesenchyme and (B) UGR, liver, and limb bud cells. The percentage of GFP^+ cells for each tissue is indicated. In the right panels the results of transplantation of these sorted cells is shown. The *x* axis of the bar charts indicates the number of embryo equivalents of each sorted cell population that was transplanted. One ee of GFP^+ cells from the aorta-mesenchyme is on average 1.6×10^3 (range 1.3 to 2.1×10^3), and one ee of GFP^- cells is 9×10^4 (range $8-10 \times 10^4$). The bars represent the percentage of recipients found positive for donor repopulation at >4 months posttransplantation. The number of recipients repopulated/total number of recipients is indicated for each bar. Donor cell engraftment, as measured by peripheral blood DNA PCR ranged from 11% to 100%.

(C) Flow cytometric analysis of peripheral blood from a normal adult *Ly-6A GFP* transgenic control and a GFP^+ aorta cell transplant recipient. GFP fluorescence is on the *x* axis, and phycoerythrin fluorescence is on the *y* axis. Directly conjugated phycoerythrin B220-, CD4-, CD8-, and Mac-1-specific antibodies were used for blood cell staining. Percentage of double-positive cells is indicated in the upper right quadrants.

(D) Hematopoietic multilineage analysis of two recipient mice repopulated with GFP^+ aorta-mesenchyme cells. At >4 months posttransplantation, DNA was isolated from peripheral blood (PB), bone marrow (BM), bone marrow myeloid cells (bM), bone marrow B cells (bB), spleen (S), spleen B cells (sB), spleen T cells (sT), thymus (Th), and lymph nodes (L). PCR analysis was performed using oligonucleotide primers for *GFP* (top band) and myogenin (*myo)* (lower band) genes. One hundred, seventy-five, fifty, twenty-five, ten, one, and zero are the contribution controls indicating percentage donor cell engraftment. Percentage engraftment by donor cells (indicated below each gel) was calculated by ImageQuant. Transplantation data are from two independent experiments.

were found to express GFP and include the liver, hindgut, and limb buds (data not shown). These GFP^+ tissues were tested for HSC activity. Flow cytometry (Figure 2B, left panels) revealed that these tissues contained between 2% and 4.2% GFP^+ cells. As shown in Figure 2B (right panels), no HSCs (GFP^+ or GFP^-) were found in the E11 UGRs or the limb buds. However, HSCs of the E11 liver were exclusively in the GFP^+ fraction. Thus, all HSCs are GFP^+ but not all GFP^+ cells are HSCs.

Ly-6A GFP Expression Is Localized to the Endothelial Cell Layer of the Midgestation Dorsal Aorta

Immunohistochemical staining was performed to establish what cell lineage(s) the GFP-expressing cells in the dorsal aorta may represent. Transverse sections through the trunkal regions of E11 *Ly-6A GFP* embryos were stained using antibodies to CD31 (PECAM-1), a marker expressed on all endothelial cells and on intraaortic hematopoietic clusters (Cai et al., 2000; Drake and Fleming, 2000; Garcia-Porrero et al., 1998), and Sca-1. Low magnification of a transverse section through the trunkal region of an E11 *Ly-6A GFP* embryo stained with CD31 shows a single layer of $CD31^+$ (red; Cy5) endothelial cells lining the entire circumference of the dorsal aorta (Figures 4A and 4B). GFP expression appears to be colocalized within a few of these endothelial cells. High magnification of the ventral area within the box shows single green and red fluorescence signals

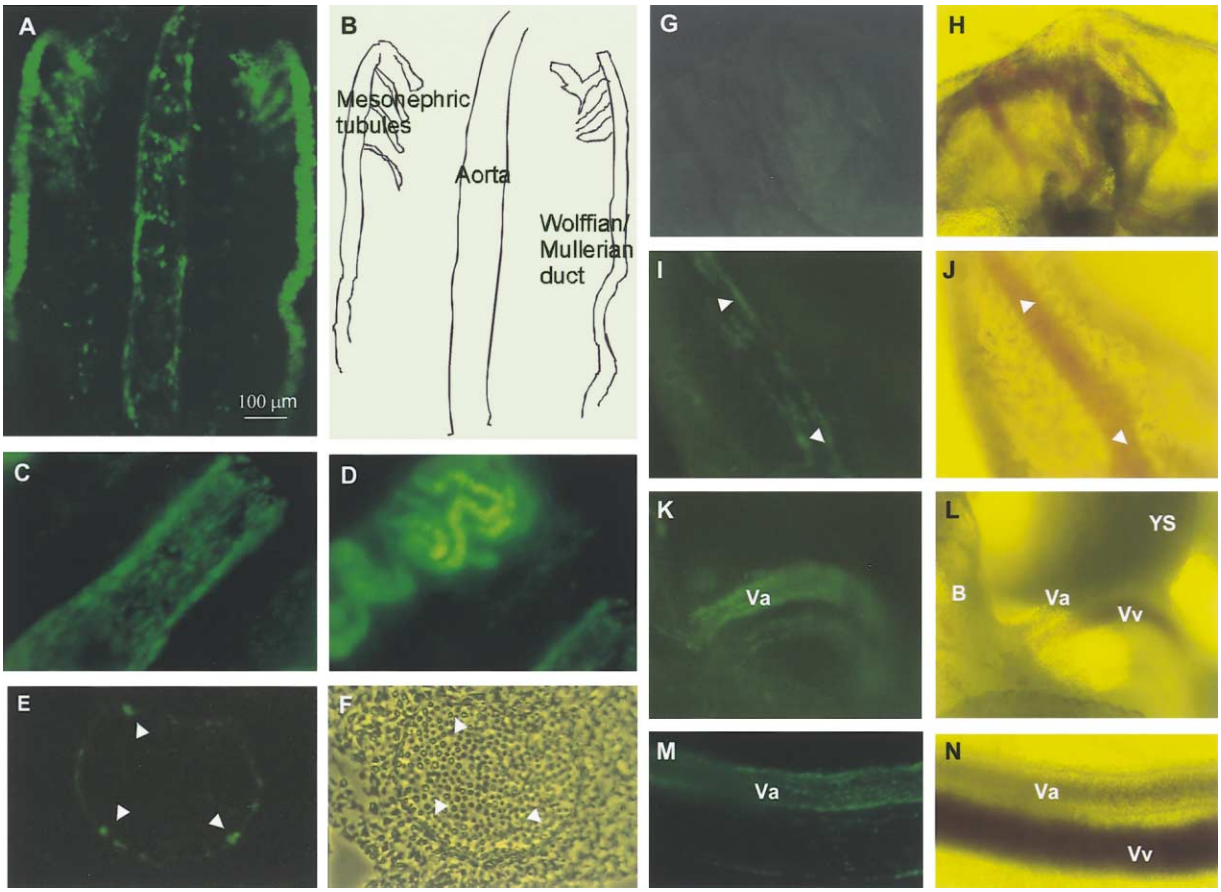

Figure 3. Fluorescent Microscopic Analysis of Whole AGM and Tissue Sections from *Ly-6A GFP* Embryos

(A) Confocal microscopic analysis of whole late E11 AGM tissue at 10× magnification. Twenty-four scanned images of this AGM region were obtained at 10 μm intervals. The scanned image in this figure is the 12th optical slice of 24 with a thickness of approximately 1 μm. All 24 images of the Z stack of this AGM were used to build the three-dimensional projection shown at www.eur.nl/fgg/ch1 (see Cell Biology).

(B) Schematic drawing of the GFP expression region of the E11 AGM shown in (A). GFP positive areas include the aorta, mesonephric tubules, and Wolffian/Mullerian ducts.

(C and D) GFP fluorescent image of a portion of the E11 aorta (C) and mesonephric tubules (D) at 20× magnification, 1 μm thickness.

(E and F) Fluorescent (E) and bright field (F) images of a transverse section of the AGM region of a 43 somite pair (sp) transgenic embryo (E11) showing the dorsal aorta. Dorsal side is up and ventral is down. A single-cell layer of interspersed GFP^+ cells is found only around the circumference of the dorsal aorta and is indicated by the arrowheads. Magnification 10×.

(G and H) Fluorescent (G) and bright field (H) images of a yolk sac from a 34 sp transgenic embryo (E10). No fluorescent signal is detected in the yolk sac, particularly the vessels. Magnification 4×.

(I and J) Fluorescent (I) and bright field (J) images of a yolk sac from a 48 sp transgenic embryo (E11). GFP signal is found in the cells lining the large vessel. Arrowheads indicate the vessel walls.

(K and L) Fluorescent (K) and bright field (L) images of the vitelline vessels as they emerge from the body wall (B) of a 38 sp transgenic embryo (E10). Strong GFP signal is observed in the vitelline artery (Va) with some low signal in the underlying vein (Vv). The fluorescent negative yolk sac (YS) is shown in the background. Magnification 4×.

(M and N) Fluorescent (M) and bright field (N) images of the vitelline vessels from an E14 transgenic embryo. The endothelial cells of the vitelline artery (Va) are strongly GFP positive. Magnification 4×.

(Figures 4E and 4F). An overlay of both signals (Figure 4G) reveals GFP expression (green) within the cytoplasm and the red signal of the CD31 antibody on the surface of a pair of double-positive cells (see arrow, Figure 4G). The two adjacent cells are located within the single layer of cells lining the aorta. A third double-positive cell is seen further to the right. Unfortunately, while we examined many of these transverse sections to evaluate the GFP expression status, we were unable to find any hematopoietic clusters. Thus, from the results presented here, GFP expression is specifically localized to a few of the cells within the single-cell layer of endothelial cells lining the dorsal aorta, and these cells express the CD31 endothelial/hematopoietic cluster marker.

Finally, the expression of GFP and endogenous Sca-1 was examined by fluorescent microscopy on transverse sections through the trunkal region of E11 *Ly-6A GFP* embryos. As shown in Figure 4C (GFP expression) and D (GFP and Sca-1 expression), Sca-1 expression clearly overlaps with the GFP signal in the mesonephric tubules. However, no (or undetectable) Sca-1 expression was found in the GFP^+ cells lining the walls of the dorsal

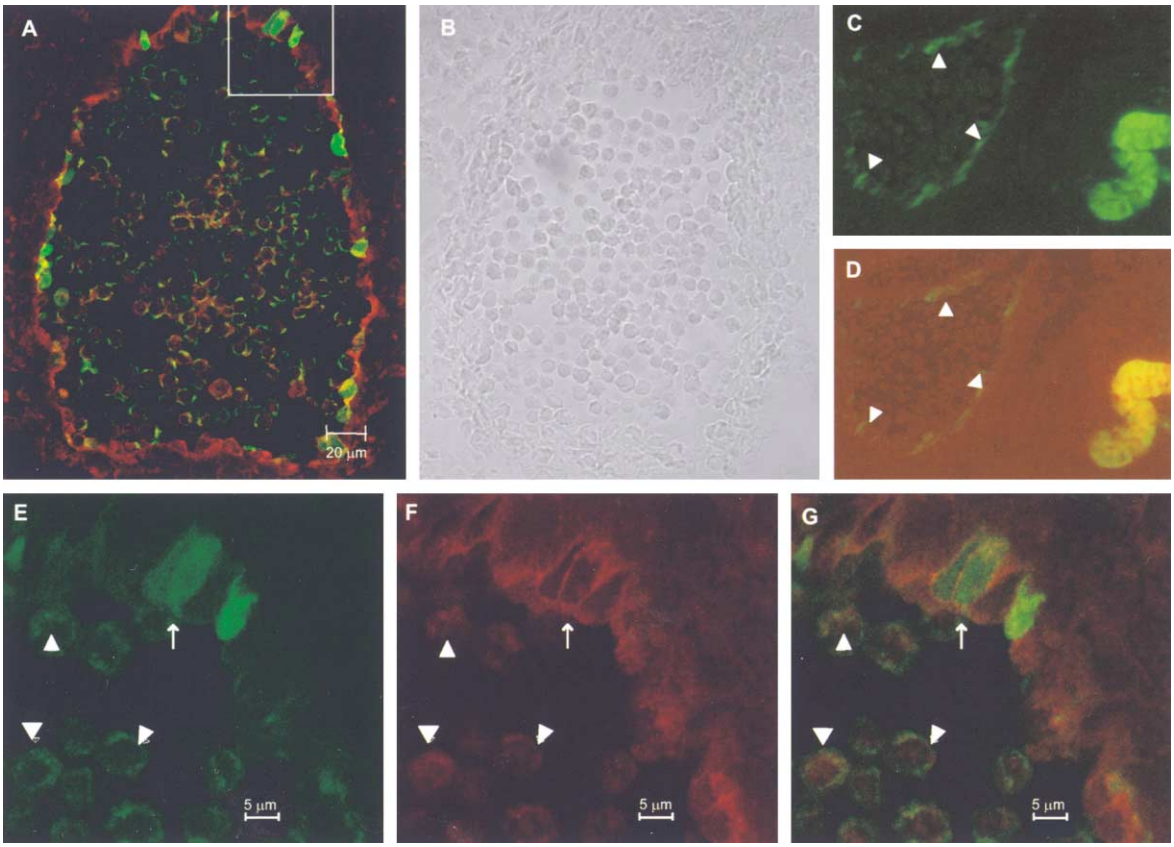

Figure 4. Fluorescence and Immunostaining Confocal Microscopic Analysis of AGM Tissue Sections from *Ly-6A GFP* Embryos

(A and B) Fluorescence microscopic image (A) and bright field image (B) of a transverse section through the dorsal aorta of an E11 (43 somite pair) embryo, showing immunofluorescent staining with an antibody against CD31 (red) and GFP transgene signal (green). Part of the ventral wall of the aorta (marked by a box) is shown at a higher magnification in (E) GFP signal, (F) CD31 Cy5 signal, and (G) an overlay of GFP and CD31 signals. The arrow indicates two adjacent cells that coexpress the *Ly-6A GFP* transgene and CD31. Arrowheads indicate some of the autofluorescent erythrocytes.

(C) Transverse section through an E11 AGM region showing a single layer of GFP^+ cells (green) around the dorsal aorta (and strongly fluorescent mesonephric tubules). Ventral is to the upper right.

(D) Sca-1 (red) and GFP immunofluorescence on section as shown in (C). Overlap in expression of Sca-1 and GFP occurs in the mesonephric tubules, but no (or undetectable) overlap is found in aorta cells. Size bars are included for (A), (E), (F), and (G), and (C) and (D) are at a 40× magnification.

(E–G) For (E), (F), and (G) (CD31 immunostaining), optical slice eight from 18 optical scans of approximately 1 μm thickness at 0.5 μm intervals is shown. All 18 optical slices, respectively, were used to build the three-dimensional projection shown at www.eur.nl/fgg/ch1 (see Cell Biology).

aorta, suggesting that either expression or the sensitivity of detection is more limited for the endogenous Sca-1 antigen than for GFP fluorescence.

GFP^+ Aorta Cells Express Additional Endothelial and Hematopoietic Markers

Since we have shown that hematopoietic and/or endothelial markers such as c-kit, CD31, CD34, and VE-cadherin are expressed on functional adult repopulating AGM HSCs from normal embryos (Sanchez et al., 1996; North et al., 2002), we performed flow cytometric analysis with antibodies specific for these markers and for Sca-1 on cells from E11 *Ly-6A GFP* aorta-mesenchyme. Flow cytometric profiles for UGRs are provided for comparison. As shown in Figure 5, a large percentage of GFP^+ cells in the UGRs are Sca-1^+ (68%) as expected from the immunostaining results showing high coexpression of Sca-1 and GFP in the mesonephric tubules. However, in the aorta-mesenchyme fewer GFP^+ cells coexpress Sca-1^+ (19%). This is in accordance with the immunohistochemical and transplantation results and suggesting that cell surface Sca-1 antigen expression is limited on cells of the aorta and particularly on HSCs.

We next examined the expression of c-kit, CD34, CD31, and VE-cadherin on cells from these subregions. Interestingly, all of these markers have been shown to be expressed in hematopoietic clusters as well as the endothelial cells lining the dorsal aorta in midgestation embryos (Bernex et al., 1996; Wood et al., 1997; Drake and Fleming, 2000; Garcia-Porrero et al., 1998; Cai et al., 2000; North et al., 2002). In the aorta-mesenchyme, c-kit^+ cells make up 37%–51% of the GFP^+ population. As all AGM HSCs are known to express c-kit, these results strongly suggest that HSCs are in this double-positive population. As expected from the endothelial localization of GFP^+ cells by microscopy, many GFP^+

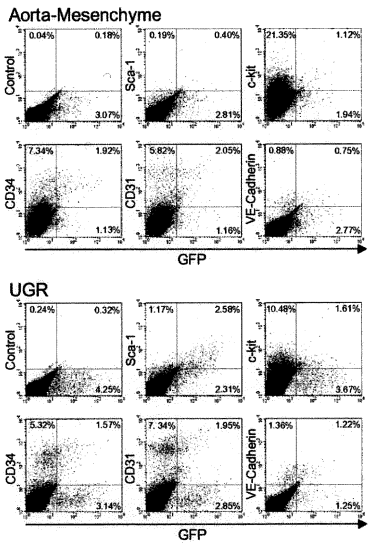

Figure 5. Representative FACS Profiles for E11 Aorta-Mesenchyme and UGR Subregions

Cells from these two AGM subregions were obtained from *Ly-6A GFP* transgenic embryos and were stained with antibodies specific for Sca-1, c-kit, CD34, CD31, and VE-cadherin antigens. Three to five times 10^4 cells were analyzed for GFP (*x* axis) and surface antigen (*y* axis) expression on a FACS Vantage SE. Dead cells were gated out from the analysis. Percentages of cells in each quadrant are indicated and are representative of the profiles obtained from two independent analyses.

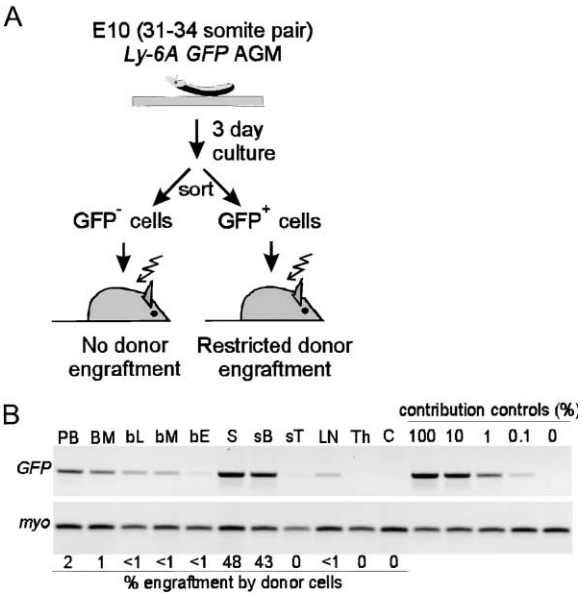

Figure 6. Hematopoietic Engraftment by Sorted GFP$^+$ Cells after Explant Culture of Early E10 *Ly-6A GFP* AGMs

(A) Experimental protocol. Twelve transgenic AGMs of the 31 to 34 somite pair stage were cultured as explants for 3 days and harvested, and the cells were sorted on the basis of GFP transgene expression. Irradiated adult recipient mice were injected with either 3 ee or 1.5 ee of GFP$^+$ (2.6×10^4 cells/ee) or GFP$^-$ (3.5×10^5 cells/ee).

(B) DNA PCR analysis, for the donor cell *GFP* transgene marker and the myogenin (*myo*) gene normalization control, showing low level, restricted hematopoietic engraftment of a recipient mouse at 6 months after injection of 3 ee of GFP$^+$ cells. Cells were obtained from each of the hematopoietic tissues indicated and from lineage-specific sorts performed using antibodies directed against B cells, T cells, monocytes/granulocytes, and erythroid cells. PB, peripheral blood; BM, bone marrow; bL, bone marrow lymphoid; bM, bone marrow myeloid; bE, bone marrow erythroid; S, spleen; sB, spleen B cells; sT, spleen T cells; LN, lymph nodes; Th, thymus; and C, control transplanted with nontransgenic cells. Contribution controls show signal expected from varying percentages of *GFP* transgene containing DNA. Percentage engraftment is indicated below each lane. Quantitation was performed using ImageQuant.

cells in the aorta-mesenchyme are also CD34$^+$, CD31$^+$, and VE-Cadherin$^+$ (63%, 64%, and 21% of all GFP$^+$ cells, respectively). In combination with our data that all normal AGM HSCs express c-kit, CD34, CD31, and VE-Cadherin (Sanchez et al., 1996; North et al., 2002), these data indicate that the first HSCs are among the GFP$^+$ cells in the aorta that coexpress these endothelial/hematopoietic markers.

Early E10 AGM Explants Contain GFP$^+$ Multipotential/Lymphoid Progenitors

Previously, we have been unable to obtain high level, multilineage hematopoietic repopulation from directly transplanted AGM regions (Muller et al., 1994) or AGM explants (Medvinsky and Dzierzak, 1996) before the 35 somite pair stage. The appearance of a few GFP$^+$ cells in the AGM region of *Ly-6A GFP* transgenic embryos beginning at the 24 somite pair stage (late E9), together with the finding that not all E11 AGM GFP$^+$ cells are HSCs, prompted us to test these early appearing GFP$^+$ cells for other hematopoietic progenitor activity. We cultured AGM explants from early E10 *Ly-6A GFP* embryos (31 to 34 somite pairs) for 3 days, sorted the GFP$^+$ and GFP$^-$ cells, and transplanted these cells into irradiated adult recipients (Figure 6A). At 4 months posttransplantation, only those animals (two out of two) transplanted with GFP$^+$ cells were positive for donor cell engraftment. Peripheral blood DNA PCR revealed levels of engraftment of 1%–2% in these animals. Multilineage analysis of hematopoietic tissues was performed at 6 months posttransplantation for one animal. Engraftment of donor-derived cells was found mainly in the peripheral blood, bone marrow, and spleen (Figure 6B). After sorting cells from the bone marrow and spleen with antibodies specific for myeloid, erythroid, and B and T lymphoid lineages, a predominance of donor-derived cells in the B lymphoid lineage was found. Only very low levels (<1%) of myeloid and erythroid lineage cells were found, suggesting that this animal was long-term repopulated with a multipotent/lymphoid progenitor. Thus, the early expression of GFP in the AGM region (before E10.5) may represent cells that are the predecessors of high level, multilineage repopulating HSCs or unrelated progenitors.

Discussion

Ly-6A GFP Marks Definitive HSC Emergence in the Dorsal Aorta and Vitelline and Umbilical Arteries

Unlike the Sca-1 surface glycoprotein, flow cytometric sorting based on GFP expression reliably enriches for all AGM HSCs. This difference is due most likely to the limiting nature of Sca-1 protein on the surface of these

HSCs compared to a more intense fluorescence signal produced by GFP. Indeed, the transgenic mice contain eight copies of the transgene as compared to the normal diploid copy of the endogenous *Ly-6A/E* gene encoding Sca-1. Furthermore, GFP is a cytoplasmic protein not requiring the processing steps necessary to get the Sca-1 protein properly displayed on the cell surface (i.e., glycosylation, transport through the plasma membrane, and GPI linkage). Thus, the *Ly-6A GFP* transgene appears to be an optimally expressed reporter in AGM HSCs and is an excellent marker for HSC isolation from the embryo.

Flow cytometric analyses show that there are approximately 1600 GFP^+ cells within the E11 aorta-mesenchyme. While in transplantation studies all E11 AGM aorta-mesenchyme HSCs are GFP^+, it is clear that not all 1600 GFP^+ aorta-mesenchyme cells are functional HSCs. Outside the aorta subregion, we also found a significant percentage of GFP^+ cells in the limb buds, tail, and UGRs. In the E11 UGRs, we show here and in a previous study (de Bruijn et al., 2000) that there are no HSCs even at high cell numbers transplanted. However, considering that HSCs can be detected in E12 UGRs or explant cultured E11 UGRs (de Bruijn et al., 2000), further studies must be performed to determine whether these HSCs are GFP^+. Recent reports on the multipotentiality of stem cells for lineages outside the expected lineage (reviewed in Wulf et al., 2001 and Morrison, 2001) led us to analyze the hematopoietic potential of the other GFP^+ sites. If indeed the *Ly-6A GFP* transgene marks more totipotent stem cells, these cells in the developing limb and tail might be able to contribute to adult hematopoietic repopulation. In support of this, Godin and colleagues (1999) found some hematopoietic colony-forming activity in limb bud tissues. When we sorted and transplanted high ee of GFP^+ limb bud cells, we found no hematopoietic repopulation. Similarly, we have transplanted high ee of cells from the tail and found no detectable adult hematopoietic repopulation (our unpublished data). However, given the low frequency of stem cells and the limited number of mice transplanted, additional studies are warranted.

Enrichment of Aorta HSCs as They Emerge during Midgestation

At E11 there is at least one adult repopulating HSC in 1600 GFP^+ aorta cells. Our FACS analysis data and transplantation results with sorted AGM cells using conventional antibody-mediated enrichment suggest that further enrichment can be achieved. For example, of the 1600 GFP^+ aorta cells, a smaller number are also positive for c-kit, CD31, CD34, or VE-cadherin, markers previously used for enrichment of AGM HSCs (Sanchez et al., 1996; North et al., 2002). Consistent with our transplantation data for the aorta-mesenchyme in which not all HSCs are Sca-1^+, more GFP^+ cells express c-kit than Sca-1. Since it has already been shown that all AGM HSCs are c-kit^+ and we have shown here that all aorta HSCs are GFP^+, a 50% enrichment can be achieved, and only 800 c-kit^+GFP^+ cells should be required for complete multilineage adult recipient repopulation. Three or four color sorting using c-kit and GFP expression together with CD34 and/or CD31 may allow an even greater enrichment of aorta HSCs. In addition, close observation of the GFP fluorescence profile of E11 aorta-mesenchyme cells reveals two populations of cells: a GFP^{high}-expressing population representing on average 0.3% of total cells and a GFP^{low}-expressing population representing 2.1% of total cells. As a means of achieving greater enrichment of aorta HSCs, we have begun to examine which population contains adult repopulating HSCs. Our preliminary results show adult repopulating HSCs within the GFP^{high} population. In the best instance this may represent a further 10-fold enrichment that, when put together with c-kit sorting, may yield one HSC in 80 c-kit^+GFP^{high} cells.

Specific Endothelial Localization of HSCs in the Midgestation Embryo

The temporal and spatial expression pattern of the *Ly-6A GFP* transgene within the expected hematopoietic sites of the midgestation mouse embryo is highly suggestive of expression in HSCs. Indeed, in our functional transplantation and fluorescence microscopy studies we have shown that all aorta-mesenchyme HSCs are GFP^+ and that they are localized to the single-cell layer lining the dorsal aorta. Furthermore, GFP expression is found in the vitelline/umbilical arteries, which together with the aorta, are the other sites within the embryo in which HSCs first appear at mid-E10. At later time points, GFP expression is found in the E11 liver (Figure 2) and E11 yolk sac (Figure 3), again coincident with the appearance of HSCs as determined in previous studies (Muller et al., 1994; Medvinsky and Dzierzak, 1996). We have shown here that all HSCs in the E11 liver are GFP^+, and in other related studies (Ma et al., unpublished data) we have found that all bone marrow HSCs in adult transgenic mice are also GFP^+. Thus, in the various spatially and temporally distinct sites tested, the *Ly-6A GFP* transgene faithfully marks all HSCs.

The identification of the direct cellular precursors of HSCs is of great importance in understanding how the adult hematopoietic system is generated. Transverse sections through the E11 dorsal aorta reveal GFP^+ cells only in the single layer of $CD31^+$ endothelial cells. No GFP^+ cells were found within the mesenchymal tissue immediately surrounding and flanking the dorsal aorta. Indeed, functional adult HSCs at this stage are $CD31^+$ (North et al., 2002). Thus, our data strongly suggest that HSCs are generated within the single layer of endothelial cells lining the wall of the dorsal aorta and possess both endothelial and hematopoietic cell surface characteristics.

At early E10, GFP expression in the aorta is limited to the ventral endothelium, the area in which many investigators have demonstrated emerging hematopoietic clusters. On the basis of our data we cannot make the distinction between the aortic endothelium or the hematopoietic clusters as the site of HSC emergence since we have not been able to find any clusters in sections from numerous GFP^+ embryos analyzed. Despite our inability to find clusters, if such cells were GFP^+ we would postulate that either these cells, the GFP^+ cells within the endothelial layer, or both cell populations are the functional adult repopulating HSCs. Indeed, our recent findings using combinations of antibodies discern-

ing hematopoietic, endothelial, and mesenchymal cells in the wild-type E11 AGM region demonstrate that the HSCs exhibit not only a hematopoietic but also an endothelial surface phenotypic profile (North et al., 2002). Taken together, these results strongly suggest that adult repopulating HSC activity normally arises within the endothelial and/or hematopoietic cluster cells and not in the mesenchymal cell layer of the dorsal aorta. It is of interest to note that North and colleagues revealed a population of adult-type HSCs in *Runx1* haploinsufficient embryos with a mesenchymal surface phenotype, which is absent from normal embryos, indicating that a full dose of *Runx1* is required for the normal development of the adult repopulating HSCs in the aorta (North et al., 2002).

Finally, we have shown by explant culture of early E10 AGM regions and subsequent GFP sorting and transplantation that an earlier restricted hematopoietic progenitor is marked by the *Ly-6A GFP* transgene. Similar low level, adult repopulating cells in the AGM region have been recently reported by Cumano and colleagues (2001), and others have reported neonatal repopulating hematopoietic cells in the AGM (Yoder et al., 1997) at time points earlier than E10.5 onset of HSC function. Here we have observed the appearance of GFP expression in the AGM region and the vitelline/umbilical arteries at times earlier than E10.5. Hence, these *Ly-6A GFP*-expressing cells may represent the direct precursors to the high level adult repopulating cells or alternatively represent the differentiation products of the same or similar endothelial cohort that gives rise to the HSCs. Interestingly, an abundance of GFP^{low}-expressing cells were found in the explant cultures of early E10 AGM regions, and future sorting and transplantion experiments will examine whether these restricted low level repopulating cells are correlated with GFP expression levels.

In summary, we have shown here by transplantation and immunofluorescence analyses of midgestational *Ly-6A GFP* transgenic mouse AGM regions that all HSCs in the aorta are GFP^+ and that GFP-expressing cells are localized to the endothelial layer of the dorsal aorta but not the underlying mesenchyme. This localization can now lead to an identification of the immediate precursors to HSCs and a further understanding of the molecular signals necessary for the emergence of these somatic stem cells.

Experimental Procedures

Transgenic Mice and Embryo Generation

Timed matings were set up between *Ly-6A-GFP*, BL1b (Miles et al., 1997), or line 72 (Strouboulis et al., 1992) transgenic male mice and wild-type (C57BL/10 × CBA)F_1 females. The day of vaginal plug detection is day 0. For embryo generation, the transgene was always inherited through the male parent to avoid contribution by genetically marked maternal cells in transplantation assays. Transgenic embryos were typed on the basis of their GFP expression using an Olympus IX70 fluorescent microscope. Animals were housed according to institutional guidelines, and procedures were carried out in compliance with the Standards for Humane Care and Use of Laboratory Animals.

Cell Preparations

Pregnant mice were killed by cervical dislocation. Embryos were isolated from the uterus, and AGMs were dissected and subsequently subdissected into the aorta with its surrounding mesenchyme and the UGRs using 27 gauge needles (Becton Dickinson, San Jose, CA). Tissues were either incubated directly with collagenase (type I, Sigma; final concentration 0.12% v/v) for 1 hour at 37°C in phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin or cultured as explants for 3 days prior to collagenase digestion (Medvinsky and Dzierzak, 1996). Cells were dispersed, washed, resuspended in PBS+FCS, sorted, and transplanted intravenously into irradiated mice. Viable cell numbers were determined with a Bürker hemocytometer using trypan blue. Cells were kept on ice at all times subsequent to collagenase digestion until the time of injection.

Analysis of Long-Term Multilineage Repopulating Activity

Embryonic cell suspensions were assayed for the presence of definitive HSCs by intravenous transfer into irradiated adult recipients (Dzierzak and de Bruijn, 2002; Muller et al., 1994; Miles et al., 1997; Medvinsky and Dzierzak, 1996). In brief, (C57BL/10 x CBA)F_1 male recipients were exposed to a split dose of 900 rad of γ irradiation from a ^{137}Cs source. Adult (C57BL/10 x CBA)F_1 spleen cells (2×10^5/mouse) were coinjected with the embryonic cells to promote survival. Recipient mice were bled at 1 and >4 months after transfer and analyzed for donor contribution by donor marker-specific PCR on peripheral blood DNA (Dzierzak and de Bruijn, 2002; Miles et al., 1997; Muller et al., 1994). Reconstitution was evaluated by ethidium bromide staining of agarose gels and in some cases by Southern blot hybridization as described (Dzierzak and de Bruijn, 2002; Medvinsky and Dzierzak, 1996; Muller et al., 1994). To test for long-term multilineage hematopoietic repopulation, genomic DNA was isolated from peripheral blood, thymus, lymph node, splenic B and T cells, and bone marrow myeloid, erythroid, and lymphoid cells. Percentage donor-cell contribution was analyzed by ImageQuant using the *myo* gene as the DNA normalization control.

Fluorescent Antibody Surface Staining and Flow Cytometry

All antibodies used in flow cytometric sorting and analysis were obtained from Pharmingen. The monoclonal antibodies used were directly conjugated with either phycoerythrin (PE) or biotin, or unconjugated, and included PE-anti-c-kit (clone 2B8), PE-anti-Sca-1 (Ly-6A/E; clone E13-161.7), PE-anti-CD4 (clone GK1.5), PE-anti-CD8a (clone 53-6.7), PE-anti-B220 (CD45R; clone RA3-6B2), PE-anti-Mac-1 (CD11b; clone M1/70), biotinylated CD31 (PECAM-1; clone MEC 13.3), CD34 (RAM34), and unconjugated purified VE-Cadherin (CD144; clone 11D4.1). Single-cell suspensions were prepared as described above. After incubation with specific antibodies for 30 min on ice, cells were washed twice and incubated with PE-conjugated streptavidin or PE-conjugated goat anti-rat IgG (mouse-absorbed) (Caltag Laboratories, Burlingame, CA) when required. Cells were washed twice and filtered through a 45 μm nylon mesh screen prior to sorting. Hoechst 33258 (1 μg/ml) or 2 μg/ml 7AAD was added to identify dead cells. To determine the background levels, cells were stained with appropriate immunoglobulin isotype controls (Pharmingen). During the entire staining procedure, PBS containing 10% FCS and penicillin/streptomycin was used. Cells were sorted/analyzed using a FACS Vantage SE (Becton-Dickinson). The purity of the sorted cells ranged from 80%–98%. For phenotypic analysis of stained whole blood cell samples, suspensions were treated for 10 min with FACS lysis solution (Becton-Dickinson) to eliminate erythrocytes and washed before analysis.

Immunohistochemical Staining and Confocal Microscopy

Ly-6A GFP transgenic embryos were fixed in 2% paraformaldehyde/PBS for 30 min at room temperature, equilibrated in 20% sucrose/PBS overnight at 4°C, quick frozen in Tissue Tek, and stored at −70°C until use. After cryosectioning, tissues were treated in 100% cold acetone for 10 min, washed with PBS (0.05% tween), blocked with 0.5% BSA and 50% v/v Avidin D block solution (Vector Laboratories Inc., Burlingame, CA) for 15 min, washed three times, blocked with Biotin blocking solution (Vector Laboratories, Inc.) for 15 min, and washed three times. Subsequently, sections were incubated with biotinylated antibodies for CD31 or Sca-1 (Pharmingen) at room temperature for 1 hr, washed three times, incubated with the detection reagent avidin TexasRed (Vector Laboratories Inc.) or Streptavi-

din-Cy5 (Rockland, Gilbertsville, PS), washed three times, dehydrated in ethanol (from 70% to 100%), and mounted with vectashield (Vector Laboratories, Burlingame, CA). For whole mount confocal microscopy, AGM tissues were carefully dissected from E11 *Ly-6A GFP* embryos, kept in PBS (containing 10% FCS), and directly observed.

DNA Analysis

Genomic DNA (200 ng) from the peripheral blood of transplant recipients was analyzed by PCR using oligonucleotide primers for *myogenin*, 5′TTACGTCCATCGTGGACAGC3′ and 5′TGGGCTGGGTGT TAGTCTTA3′, GFP 5′GACAGAACTTGCCACTGTGC 3′ and 5′AAG AAGATGGTGCGCTCCTG 3′, human β-*globin* 5′CTTCAGGTTCCC AGTGAGGATG3′ and 5′GCTCCCTAAGGGGTAAAGAGTG3′, and *lacZ* 5′GCGACTTCCAGTTCAACATC3′ and 5′GATGAGTTTGGACA AACCAC3′.

DNA was subjected to an initial 5 min denaturation at 94°C followed by 30 cycles of denaturation (1 min at 94°C), annealing (2 min at 58°C [β-*globin*] or 55°C [*lacZ* and *GFP*]), and elongation (2 min at 72°C). Serial dilutions of blood DNA from a transgenic animal were used as a control to evaluate the levels of donor cell reconstitution in transplanted mice.

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References

Bernex, R., De Sepulveda, P., Kress, C., Elbaz, C., Delouis, C., and Panthier, J.-J. (1996). Spatial and temporal patterns of *c-kit*-expressing cells in $W^{lacZ}/+$ and W^{lacZ}/W^{lacZ} mouse embryos. Development *122*, 3023–3033.

Cai, Z.L., de Bruijn, M., Ma, X., Dortland, B., Luteijn, T., Downing, J.R., and Dzierzak, E. (2000). Haploinsufficiency of AML1/CBFA2 affects the temporal and spatial generation of hematopoietic stem cells in the mouse embryo. Immunity *13*, 423–431.

Cumano, A., Ferraz, J.C., Klaine, M., Di Santo, J.P., and Godin, I. (2001). Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation provide long-term multilineage reconstitution. Immunity *15*, 477–485.

de Bruijn, M.R.T.R., Speck, N.A., Peeters, M.C.E., and Dzierzak, E. (2000). Definitive hematopoietic stem cells first emerge from the major arterial regions of the mouse embryo. EMBO J. *19*, 2465–2474.

Drake, C.J., and Fleming, P.A. (2000). Vasculogenesis in the day 6.5 to 9.5 mouse embryo. Blood *95*, 1671–1679.

Dzierzak, E., and de Bruijn, M. (2002). Isolation and analysis of hematopoietic stem cells from mouse embryos. In Methods in Molecular Medicine: Hematopoietic Stem Cell Protocols, C. Klug and C. Jordan, eds. (Totowa, NJ: The Humana Press Inc.), pp. 1–14.

Garcia-Porrero, J.A., Godin, I.E., and Dieterlen-Lievre, F. (1995). Potential intraembryonic hemogenic sites at pre-liver stages in the mouse. Anat. Embryol. (Berl.) *192*, 425–435.

Garcia-Porrero, J.A., Manaia, A., Jimeno, J., Lasky, L.L., Dieterlen-Lièvre, F., and Godin, I.E. (1998). Antigenic profiles of endothelial and hemopoietic lineages in murine intraembryonic hemogenic sites. Dev. Comp. Immunol. *22*, 303–319.

Godin, I., Garcia-Porrero, J.A., Dieterlen-Lievre, F., and Cumano, A. (1999). Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites. J. Exp. Med. *190*, 43–52.

Huang, H., and Auerbach, R. (1993). Identification and characterization of hematopoietic stem cells from the yolk sac of the early mouse embryo. Proc. Natl. Acad. Sci. USA *90*, 10110–10114.

Kamiura, S., Nolan, C.M., and Meruelo, D. (1992). Long-range physical map of the Ly-6 complex: mapping the Ly-6 multigene family by field-inversion and two-dimensional gel electrophoresis. Genomics *12*, 89–105.

Khan, K.D., Lindwall, G., Maher, S.E., and Bothwell, A.L. (1990). Characterization of promoter elements of an interferon-inducible Ly-6E/A differentiation antigen, which is expressed on activated T cells and hematopoietic stem cells. Mol. Cell. Biol. *10*, 5150–5159.

Khan, K.D., Shuai, K., Lindwall, G., Maher, S.E., Darnell, J.E., and Bothwell, A.L. (1993). Induction of the Ly-6A/E gene by interferon alpha/beta and gamma requires a DNA element to which a tyrosine-phosphorylated 91-kDa protein binds. Proc. Natl. Acad. Sci. USA *90*, 6806–6810.

Kimura, S., Tada, N., Liu-Lam, Y., and Hammerling, U. (1984). Studies of the mouse Ly-6 alloantigen system. II. Complexities of the Ly-6 region. Immunogenetics *20*, 47–56.

LeClair, K.P., Palfree, R.G., Flood, P.M., Hammerling, U., and Bothwell, A. (1986). Isolation of a murine Ly-6 cDNA reveals a new multigene family. EMBO J. *5*, 3227–3234.

Lemischka, I.R. (1991). Clonal, in vivo behavior of the totipotent hematopoietic stem cell. Semin. Immunol. *3*, 349–355.

Ma, X., Ling, K.-W., and Dzierzak, E. (2001). Cloning of the Ly-6A (Sca-1) gene locus and identification of a 3′ distal fragment responsible for high level γ-interferon-induced expression in vitro. Br. J. Haematol. *114*, 724–730.

Ma, X., de Bruijn, M., Robin, C., Peeters, M., Kong-A-San, J., Snoijs, C., and Dzierzak, E. (2002). Expression of the Ly-6A (Sca-1) lacZ transgene in mouse hematopoietic stem cells and embryos. Br. J. Haematol. *116*, 401–408.

Marshall, C.J., and Thrasher, A.J. (2001). The embryonic origins of human hematopoiesis. Br. J. Haematol. *112*, 838–850.

McGrew, J.T., and Rock, K.L. (1991). Isolation, expression, and sequence of the TAP/Ly-6A.2 chromosomal gene. J. Immunol. *146*, 3633–3638.

Medvinsky, A., and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. Cell *86*, 897–906.

Miles, C., Sanchez, M.-J., Sinclair, A., and Dzierzak, E. (1997). Expression of the Ly-6E.1 (Sca-1) transgene in adult hematopoietic stem cells and the developing mouse embryo. Development *124*, 537–547.

Morrison, S.J. (2001). Stem cell potential: can anything make anything? Curr. Biol. *11*, R7–R9.

Mukouyama, Y.-S., Chiba, N., Hara, T., Okada, H., Ito, Y., Kanamaru, R., Miyajima, A., Satake, M., and Watanabe, T. (2000). The AML1 transcription factor functions to develop and maintain hematogenic precursor cells in the embryonic aorta-gonad-mesonephros region. Dev. Biol. *220*, 27–36.

Muller, A.M., Medvinsky, A., Strouboulis, J., Grosveld, F., and Dzierzak, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. Immunity *1*, 291–301.

Murray, P. (1932). The development *in vitro* of the blood of the early chick embryo. Proc. R. Soc. Lond. B Biol. Sci. *11*, 497–521.

North, T., Gu, T.-L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marin-Padilla, M., and Speck, N. (1999). Cbfα is required for the formation of intraaortic hematopoietic clusters. Development *126*, 2563–2575.

North, T.E., de Bruijn, M.F.T.R., Stacy, T., Talebian, L., Lind, E., Robin, C., Binder, M., Dzierzak, E., and Speck, N.A. (2002). Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. Immunity *16*, this issue, 661–672.

Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Miura, Y., and Suda, T. (1992). In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood *80*, 3044–3050.

Okuda, T., van Deursen, J., Hiebert, S.W., Grosveld, G., and Down-

ing, J.R. (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell *84*, 321–330.

Sanchez, M.J., Holmes, A., Miles, C., and Dzierzak, E. (1996). Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. Immunity *5*, 513–525.

Shalaby, F., Ho, J., Stanford, W.L., Fischer, K.D., Schuh, A.C., Schwartz, L., Bernstein, A., and Rossant, J. (1997). A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell *89*, 981–990.

Sinclair, A.M., and Dzierzak, E.A. (1993). Cloning of the complete Ly-6E.1 gene and identification of DNase I hypersensitive sites corresponding to expression in hematopoietic cells. Blood *82*, 3052–3062.

Sinclair, A., Daly, B., and Dzierzak, E. (1996). The Ly-6E.1 (Sca-1) gene requires a 3′ chromatin-dependent region for high level gamma-interferon-induced hematopoietic cell expression. Blood *87*, 2750–2761.

Spangrude, G.J., and Brooks, D.M. (1993). Mouse strain variability in the expression of the hematopoietic stem cell antigen Ly-6A/E by bone marrow cells. Blood *82*, 3327–3332.

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science *241*, 58–62.

Spangrude, G.J., Smith, L., Uchida, N., Ikuta, K., Heimfeld, S., Friedman, J., and Weissman, I.L. (1991). Mouse hematopoietic stem cells. Blood *78*, 1395–1402.

Stanford, W.L., Bruyns, E., and Snodgrass, H.R. (1992). The isolation and sequence of the chromosomal gene and regulatory regions of Ly-6A.2. Immunogenetics *35*, 408–411.

Strouboulis, J., Dillon, N., and Grosveld, F. (1992). Developmental regulation of a complete 70-kb human beta globin locus in transgenic mice. Genes Dev. *6*, 1857–1864.

Tavian, M., Hallais, M.F., and Peault, B. (1999). Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo, Development *126*, 793–803.

van de Rijn, M., Heimfeld, S., Spangrude, G.J., and Weissman, I.L. (1989). Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc. Natl. Acad. Sci. USA *86*, 4634–4638.

Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A.H., and Speck, N.A. (1996). Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc. Natl. Acad. Sci. USA *93*, 3444–3449.

Wood, H.B., May, G., Healy, L., Enver, T., and Morriss-Kay, G.M. (1997). CD34 expression patterns during early mouse development are related to modes of blood vessel formation and reveal additional sites of hematopoiesis. Blood *90*, 2300–2311.

Wulf, G.G., Jackson, K.A., and Goodell, M.A. (2001). Somatic stem cell plasticity: current evidence and emerging concepts. Exp. Hematol. *29*, 1361–1370.

Yoder, M.C., Hiatt, K., Dutt, P., Mukherjee, P., Bodine, D.M., and Orlic, D. (1997). Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. Immunity *7*, 335–344.

Note Added in Proof

We have recently found some rare GFP^+ clusters on the ventral wall of the dorsal aorta in transverse sections from E10 *Ly-6A GFP* transgenic embryos.