Embryonic beginnings of adult hematopoietic stem cells

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Hematopoietic stem cells (HSC) are at the foundation of the adult hematopoietic system. HSC give rise to all blood cells through a complex series of proliferation and differentiation events that occur throughout the lifespan of the individual. Because of their clinical importance in transplantation protocols, recent research has focused on the developmental origins and potential of embryonic HSC. In both mammalian and non-mammalian vertebrate embryos, two independent anatomical sites have been found to generate hematopoietic cells. The yolk sac (or its equivalent in amphibians, the ventral blood islands) participates in a first transient wave of hematopoiesis by producing primitive erythrocytes. Importantly, adult-type HSCs emerge autonomously in a second wave of hematopoietic generation in an intraembryonic region surrounding the dorsal aorta, the aorta-gonads-mesonephros (AGM) region. In this review, we will discuss research advances in the field of developmental hematopoiesis, with a particular emphasis on the cellular origins of AGM HSC and their regulation by the embryonic hematopoietic microenvironment.

Key words: hematopoietic stem cells, development, aorta-gonads-mesonephros (AGM) region, endothelial cells, hemangioblasts, stromal cells, microenvironment.

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The adult hematopoietic system is composed of a progressive series of differentiating pluripotent, multipotent and unipotent cellular intermediates leading to functionally distinct mature blood cell types. Throughout this hierarchy, the cell intermediates undergo extensive proliferation to generate sufficient numbers of differentiated cells necessary for the steady state maintenance of the hematopoietic system. Rare pluripotent hematopoietic stem cells (HSC) provide the foundation of this cellular differentiation hierarchy and are maintained in constant numbers in the adult bone marrow in a relatively quiescent state.

Cells within the hematopoietic hierarchy, including HSC, have been defined functionally by *in vitro* and *in vivo* assays that measure proliferation and lineage differentiation. In the mouse the most stringent assay, *in vivo* engraftment in hematopoietically compromised recipient adults, has been used to demonstrate the presence of pluripotent HSC in bone marrow (BM) and fetal liver[1,2] and several embryonic tissues.[3-6] *In vivo* transplantation of HSC into adult recipient mice depleted of endogenous HSC by high dose irradiation leads to the complete, long-term engraftment of all blood lineages by donor-derived stem cells. Thus, pluripotent HSC possess the following complex characteristics: (i) differentiation potential for all hematopoietic lineages as demonstrated by clonal markers; (ii) high proliferative potential leading to as much as 100% donor-derived hematopoietic engraftment; (iii) long-term activity throughout the lifespan of the individual; and (iv) self-renewal as demonstrated by *in vivo* serial transplantations. And, although these

stem cells have been phenotypically characterized for cell surface molecule expression, the gold standard in defining a HSC is only through *in vivo* transplantation.

The clinical importance of human HSC with such characteristics is widely recognized in transplantation scenarios for blood-related genetic deficiencies and leukemias and, hence, studies into the biology of HSC, their lineage restriction and their *ex vivo* expansion are of intense interest. Current studies on the development of hematopoietic cells in both non-mammalian and mammalian embryos are beginning to provide unique insights into the origins and induction of HSC and their potential as the founder stem cells of the adult hematopoietic hierarchy.

Developmental origins of adult hematopoietic cells in non-mammalian vertebrates

Historically, avian and amphibian species have been shown to be excellent animal models for the study of the embryonic origins of the adult hematopoietic system[7-9] and have provided a strong basis for our understanding of mammalian hematopoietic origins. The *ex utero* development of non-mammalian embryos allows for grafting and marking strategies to be used in determining the presumptive fate of embryonic cells and tissues. This is particularly important since the hematopoietic system by its very nature is in a constant state of movement through the circulation. Hence, the origins of the adult hematopoietic system can be determined most accurately at early embryonic stages before the onset of hematopoietic circulation through the creation of chimeric bird or frog embryos.[10]

Hematopoietic cells are derived from the mesodermal germ layer. Beginning shortly after gastrulation, the first hematopoietic cells observed in the conceptus are primitive erythroid cells in the avian yolk sac (YS) blood islands or the ventral blood islands (VBI) in the equivalent extra-embryonic mesodermal site in amphibians. Subsequently, hematopoietic cells are found in the intra-embryonic mesodermal site surrounding the dorsal aorta and thereafter, in other secondary tissues, such as the kidney, liver and/or bone marrow. These secondary tissues are thought to be reservoirs for the long-term maintenance of the adult hematopoietic system and are not considered to be the founding source of the self-renewing stem cells at the base of the hierarchy of adult hematopoiesis.

Orthotopic grafting experiments performed on avian or amphibian embryos[7,8] provided proof that the adult hematopoietic system was derived predominantly from founder cells in the intra-embryonic and not the extra-embryonic mesoderm. For example, when a quail embryo was grafted onto a chick yolk sac, only quail hematopoietic cells were found in the adult hematopoietic system. These results, together with the results of experiments performed on amphibian embryos, show that while the YS or VBI produce the first hematopoietic cells, these are transient blood cells present only during embryonic stages. The long-lived adult hematopoietic system is generated instead by the cells in an intra-embryonic site encompassing the dorsal aorta and pro/mesonephros (called the dorsal lateral plate, DLP in amphibians). Recent experiments in amphibian embryos have confirmed these results and more finely mapped the origins of the founder cells of the adult hematopoietic system.[9] Using molecular markers injected into single blastomeres of the 32-cell stage *Xenopus* embryo, Ciau-Utiz and colleagues were able to demonstrate that the cells of the presumptive VBI and intraembryonic DLP hematopoiesis are distinct and separated even at this early stage. Thus, the embryonic and adult hematopoietic systems are independently generated in at least two (and maybe more) distinct sites of hematopoietic development. These experiments set an important paradigm for non-mammalian vertebrate hematopoietic development.

Mouse HSC development

Early studies in the mouse embryo seemed to yield another view on the origins of the adult hematopoietic system in mammalian vertebrates. Since the *in utero* development of mammalian vertebrates precludes the use of grafting strategies for fate mapping, studies in mammalian embryos have relied on *in vitro* and *in vivo* assays to reveal the presence of an array of hematopoietic progenitors and stem cells during development. In studies pioneered in 1970, Moore and Metcalf showed that primitive erythroid cells, erythroid-myeloid progenitors, colony-forming unit-spleen CFU-S and HSC are present in the yolk sac at E7, E8, E8.5 and E11 respectively.[3] Together with limited results suggesting the absence of intra-embryonic hematopoiesis, and the assumption that mammalian hematopoietic development is different from that in non-mammalian vertebrate species, it became widely accepted that the yolk sac was the source of the adult mammalian hematopoietic system. This notion persisted for over twenty years until more extensive functional analyses were performed on the intra-embryonic mesodermally-derived tissues in the region of the dorsal aorta.

The intra-embryonic AGM region contains hematopoietic progenitors and stem cells. Two research groups independently identified, by functional analysis of sub-dissected mid-gestation mouse embryos, the presence of intra-embryonic hematopoietic activity (Figure 1). One group identified CFU-S in the region encompassing the mid-gestation dorsal aorta,

gonads and mesonephros (AGM)[11] and the other group identified lympho/hematopoietic progenitors in the para-aortic splanchnopleura (PAS, the tissue from which the more differentiated AGM is derived).[12] At E9 CFU-S activity appears in the AGM and is also present in the yolk sac. At E10 CFU-S activity in the AGM region surpasses that in the yolk sac and peaks at late E10. Thereafter, CFU-S decrease rapidly in the AGM with a concomitant increase in CFU-S in the liver, suggesting that CFU-S from the AGM region colonize the liver. Concerning the lympho/hematopoietic progenitor, at E8.5 the PAS contains progenitors for the Bla subset of B lymphocytes, as shown by transplantation of the PAS under the kidney capsule of mice with severe combined immunodeficiency (SCID). Furthermore in a two-step *in vitro* culture system, multipotent progenitors for the B and T lymphoid lineages as well as erythroid-myeloid lineages were found in the PAS at E8.5.[13] These cells were found to be positive for the AA4.1 surface marker, previously found to be on fetal liver hematopoietic progenitors and stem cells.[14] Hence, the intra-embryonic PAS/AGM region contains potent hematopoietic activity at the time when hematopoietic activity is found in the yolk sac

With these convincing data, long-term mouse radiation chimeras were generated to test whether the AGM region contains pluripotent adult-type HSC.[4] No donor-derived engraftment was observed for E8 or E9 yolk sac or AGM.[4,15] However, E10.5 AGM cells were able to engraft adult recipients fully, while E10.5 yolk sac resulted in no engraftment. Secondary and tertiary serial transplantations of bone marrow from these recipients indicated that the E10 AGM HSC are self-renewing. Moreover, at E11 the AGM region contains a high number of such HSC, with yolk sac and liver only showing HSC activity at late E11.[16] Interestingly, at E9 both the yolk sac and AGM region contain multipotent progenitors that, *in vivo*, can repopulate neonatal but not adult recipient mice.[17,18] More recently, it has been found that low-level adult repopulating cells can also be detected by transplantation into Rag-γc mutant mice.[19] Hence, the intra-embryonic PAS/AGM region contains many different hematopoietic progenitors, with the complex functional adult repopulating HSC appearing only at E10.5, subsequent to appearance of these other less potent progenitors.

The AGM region autonomously initiates HSC activity. At E10 the AGM region contains the first HSC at limiting numbers.[4] Since the circulation between the yolk sac and the embryo body is established at E8.5 in gestation,[20,21] it is possible that the AGM HSC are generated at another site within the mouse conceptus and quickly migrate through the circulation to localize in the AGM region. Thus, to

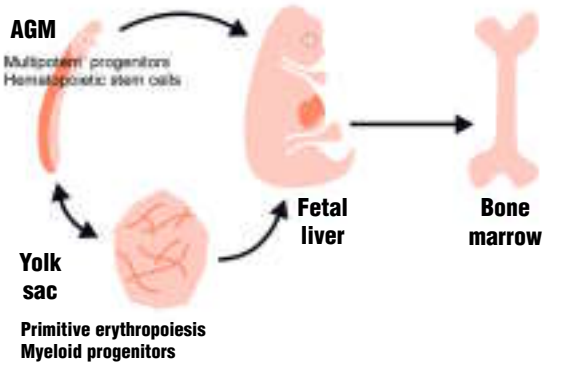

Figure 1. Hematopoietic sites during development. A proposed model for hematopoietic cell generation and colonization within the mouse embryo shows the two distinct anatomical tissues which generate hematopoietic cells, the yolk sac and the AGM (aorta-gonad-mesonephros) region, and two distinct tissues which harbor these cells at later stages, the fetal liver and adult bone marrow. The yolk sac produces primitive erythrocytes and myeloid progenitors and the AGM region produces the first multipotent progenitors and hematopoietic stem cells. Subsequent to the induction of these cells, it is thought that some of these cells are dispersed through the circulation (and/or interstitial migration) to the fetal liver and at birth to the bone marrow. The double headed arrow indicates the possible movements of hematopoietic cells between the AGM and yolk sac.

examine the site of initiation of the first HSC in the absence of circulation, organ explant cultures of AGM, yolk sac or liver were performed before *in vivo* transplantation.[5,16] The first HSC appeared in E10 cultured AGM explants and quantitatively these stem cells outnumbered those found in uncultured AGM by a factor of 15. No HSC were found in explants of E9 AGM, yolk sac or liver or E10 yolk sac or liver. HSC activity was found at late E11 in explants of yolk sac and liver. These studies demonstrate that the AGM region autonomously generates the first HSC. Since this type of study can only determine the site of the first appearance of HSC, it is uncertain whether the yolk sac also has the capacity to generate HSC autonomously. However, a recent quantitative analysis of HSC numbers in these tissues and the circulation of mid-gestation mice, suggests that the yolk sac also generates HSC that contribute to the pool of stem cells colonizing the fetal liver.[16] Nonetheless, the intra-embryonic AGM region appears to be the most potent site, containing quantitatively more HSC than the yolk sac (Figure 1).

Interestingly, when a similar organ culture step was used to determine the site of generation of the first multipotent lympho/hematopoietic progenitors, the intra-embryonic PAS region again appeared to be more potent than the yolk sac.[22] At E7.5 these progenitors are found only in the PAS. Only after the circulation between the embryo body and the yolk sac is established at E8.5 are such progenitors found in the

yolk sac. These investigations were more recently extended to the Rag-γc repopulating cell, which was again found in the PAS region before appearing in the yolk sac.[19] Thus, these definitive hematopoietic progenitors as well as fully competent adult-type HSC are autonomously generated first in the intra-embryonic PAS/AGM, before their appearance in the yolk sac (Figure 1). Taken together, these studies suggest that there are at least two independent anatomical sites, the yolk sac and the PAS/AGM, in which cells become destined to the hematopoietic lineage.

What are the direct precursors of HSC in the embryo?

The first HSC are localized to the major vasculature at mid-gestation. Recent analyses within the defined hematopoietic sites of the embryo have focused on the identification of the precusor cells that are destined to become HSC and hematopoietic progenitors. Such precursors are thought to be hemangioblasts (the common mesodermal precursor for both hematopoietic and endothelial lineages), hemogenic endothelial cells, or already fated hematopoietic cells (such as the low level, *in vivo* neonatal or Rag-γc repopulating cells). Phenotypic and functional characterization of adult repopulating AGM HSC has defined them as c-kit$^+$CD34$^+$.[23] Since E9 yolk sac and AGM cells that possess the ability to repopulate conditioned neonatal mice (but not adult recipients) are also c-kit$^+$CD34$^+$,[17] it is possible that these are the direct HSC precursors. However, since many surface markers overlap between endothelial and hematopoietic cells, such precursors could also be characterized as hemangioblasts. Indeed, gene targeting studies on the flk-1 receptor tyrosine kinase[24,25] suggest a close association between endothelial and hematopoietic cells in the mouse embryo. Both tissues are severely disrupted in flk-1 deficient mice. Furthermore, other markers and gene targeting studies support this notion.[26] In addition, several studies have demonstrated that putative endothelial cells from early stage embryos and ES cell cultures possess hematopoietic activity.[27-29] Strong support for such hemogenic endothelial cells comes from many studies demonstrating the presence of hematopoietic clusters on the floor of the dorsal aorta in species ranging from amphibians through to humans.[9,10,30-32] Some cells in these clusters have tight junctions with endothelial cells in a disrupted region of lumen.[31,32] Indeed fate mapping experiments in chick embryos demonstrate an endothelial to hematopoietic transition.[33] Lipophilic dye was used to mark aortic endothelial cells before the onset of hematopoietic cell formation. After one day of development, hematopoietic clusters were found to be dye positive, strongly supporting the notion of hemogenic endothelium as the direct precursor cell type. In the mouse, more recent studies have focused on the flow cytometric sorting and immunostaining of the AGM with antibodies directed to hematopoietic, endothelial and/or mesenchymal markers to morphologically identify emerging HSC.

Using Runx1 lacZ knock-in embryos (Runx1 is a transcription factor required for HSC generation in the mouse embryos, see below),[32,34] all HSC in the AGM and vitelline/umbilical arteries could be sorted based on the expression of β-galactosidase.[35] In such E10/E11 embryos, β-galactosidase expression is localized to hematopoietic clusters, endothelial cells and some mesenchymal cells on the ventral aspect of the dorsal aorta. Further characterization of the β-galactosidase-positive population containing HSC activity was provided by flow cytometric sorting with antibody combinations discriminating the three populations; hematopoietic, endothelial or mesenchymal. Immunostaining demonstrated the localized expression pattern of these molecules in the specific lineages. HSC activity was found in AGM cells sorted for hematopoietic cluster markers CD45$^+$ CD31$^+$ and CD45$^+$VE-cadherin$^+$, endothelial markers CD45$^-$ CD31$^+$ and CD45$^-$ VE$^-$ cadherin$^+$ and mesenchymal markers Runx1 lacZ$^+$CD45$^-$ VE$^-$ cadherin-. Thus, all three Runx1 lacZ-positive populations contain HSC activity. Interestingly, no HSC activity was found in CD45$^-$VE$^-$cadherin$^-$ AGM populations in wild type embryos. Indeed, Runx1 haploinsufficiency is known to affect the temporal and spatial generation of HSC.[34] Thus, these elegant data indicate that most HSC activity is found in the hematopoietic clusters and endothelium, with only limited activity in rapid transition in the mesenchyme (Figure 2).

Another study in mouse embryos also strongly supports the notion of hemogenic endothelium. Sca-1 is a well-known marker of bone marrow[36] and fetal liver[37] HSC. Sca-1 is also expressed on some E11 AGM HSC[35,38] and hence, since it may be a distinctive marker of adult repopulating HSC as they emerge in the embryo, a transgenic mouse expressing the *GFP* reporter gene in Sca-1 (Ly-6A)-expressing cells was produced.[39] Flow cytometric sorting and transplantation of GFP$^-$ and GFP$^+$ aorta cells into adult recipients demonstrates that all HSC of the mid-gestation AGM are Ly-6A GFP$^+$.[38] Morphologic examination of the whole and serially sectioned AGM region shows that Ly-6A GFP$^+$ cells are localized within endothelium of the dorsal aorta. Ly-6A GFP$^+$ cells are also found in the endothelium of the major vasculature (proximal vitelline and umbilical arteries). No GFP$^+$ cells are found in the mesenchyme. Most recently, some GFP$^+$ cells were also found in hematopoietic clusters. Taken together, these results strongly suggest a vascular endothelial origin, not mesenchymal origin, for the first functional adult repopulating HSC (Figure 2).

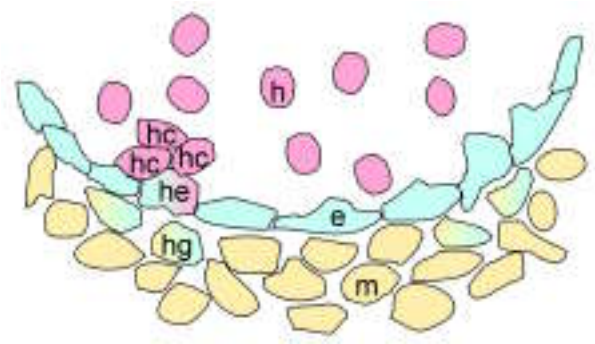

Figure 2. A schematic transverse section through an embryonic day 10 mouse aorta. Three cell lineages are depicted; hematopoietic (h) in rose color, endothelial (e) in blue and mesenchymal (m) in yellow. The direct precursors to hematopoietic cells have been thought to be hemangioblasts (hg), hemogenic endothelial cells (he), or hematopoietic cells found in clusters (hc) along the ventral wall of the dorsal aorta. Recent evidence in the mouse embryo strongly suggests that the direct precursors to hematopoietic stem cells are the hemogenic endothelial cells on the ventral wall and probably not the underlying mesenchymal (hemangioblast) cells.

Although the existence of the hemangioblast was thought to be restricted to embryonic life, results of recent studies have implied a close relationship between hematopoietic and endothelial lineages in the adult. For example, human hematopoietic cells, purified from the cord-blood or BM on the basis of CD34 and KDR (VEGF-R2) expression, generate both hematopoietic and endothelial cells *in vitro*.[40] Similarly, transplantation experiments in mice reveal that BM cells enriched in stem cell activity (expressing the Sca-1$^+$ c-kit$^+$ Lin$^-$ phenotype) are able to differentiate *in vivo* to endothelial cells in several tissues.[41]

The genetic program involved in HSC emergence

The emergence of hematopoietic cells in two distinct and separate anatomical sites, the yolk sac and embryo body, along with the emergence of functionally different embryonic and adult hematopoietic cells within the conceptus is reflected in the genetic program.[26,42] Targeted mutagenesis in mice has yielded results demonstrating differential requirements for some genes (*Runx1*, *GATA-2*, *GATA-3*, *c-kit*) in fetal liver hematopoiesis as compared to primitive yolk sac hematopoiesis. In contrast, the mutation of other genes (*flk-1*, *tal-l/SCL*) results in the impairment of both yolk sac and fetal liver hematopoiesis. Thus, the genetic programs of embryonic and adult hematopoietic cells appear to be initially overlapping during stages determining hematopoietic fate, but become unique as more complex programming is required in cells destined to become part of the adult hematopoietic system. It may be predicted that the primary events affecting fetal liver hematopoiesis occur during the induction, expansion or maintenance of HSC and progenitors in the PAS/AGM region before they colonize the fetal liver.

The Runx1 transcription factor (also known as Cbfa2 and AML-1) plays a particularly important role in HSC as the cells emerge in the AGM region. The absence of Runx1 results in embryonic death at E12.5 and profound fetal liver anemia.[43,44] *Runx1* homozygous mutant embryos show a complete absence of functional adult repopulating HSC from the earliest time of the emergence in the AGM region. Furthermore, no hematopoietic clusters are formed in the major arteries.[32] In addition, studies with *Runx1* haploinsufficient embryonic tissues suggest that HSC within the AGM region are exquisitely sensitive to the dosage of Runx1.[34] Half the dose of *Runx1* results in an earlier onset of HSC appearance in both the AGM and yolk sac. Furthermore, HSC activity becomes prematurely extinguished in cultured AGM explants suggesting a role for Runx1 in HSC generation, localization and/or maintenance. It will be interesting to determine whether this defect in such haploinsufficient AGM can be rescued by addition of specific growth factors known to be regulated by the Runx1 transcription factor. Furthermore, since the transduction of Runx1 into deficient cultured PAS cells allows endothelial cell growth but not rescue of the hematopoietic deficiency, Runx1 most likely acts at early stages of hematopoietic fate determination.[45] This is of particular interest with respect to the hematopoietic microenvironments present within the mid-gestation embryo and would support the notion of different genetic requirements for the emergence of different hematopoietic activities in specific embryonic sites.

The role of the microenvironment in the regulation of the first definitive HSC

In the adult bone marrow, HSC and their progeny develop in close association to stromal cells. These stromal cells include cells of mesenchymal origin (e.g. fibroblasts, osteocytes, adipocytes and vascular smooth muscle cells) and are known to constitute a supportive microenvironment for the survival, proliferation and differentiation of hematopoietic cells.[2] Whereas the specific role of each stromal population in the homeostasis of the HSC pool is still unknown, two recent studies clearly revealed that osteoblasts are an important component of the bone marrow HSC niche.[46,47] Initially, *in vivo* studies, using genetically deficient mice or transplantation of marrow fibroblast colonies, elegantly demonstrated the role of the microenvironment in the regulation of HSC and hematopoietic progenitors.[48,49] Furthermore, the development of *in vitro* long-term cultures and the establishment of bone marrow and fetal liver-derived

stromal cell lines demonstrated the supportive function of the stroma in the maintenance and the differentiation of HSC.[50-52] Moore and colleagues isolated and characterized a widely-used fetal liver stromal cell line, AFT024, that efficiently maintains repopulating HSC for at least 7 weeks in long-term culture.[52] Comparison of the functional properties and the genetic program of this HSC supportive stromal line with non-supportive stromal cell lines offers an exceptional tool to dissect the mechanisms involved in the cross-talk between HSC and stromal cells both at the cellular and molecular levels. Hackney *et al.* recently compared the expression profile of HSC supportive and non-supportive stromal cells by microarray screening. Bioinformatic analysis revealed that the molecular signature of the fetal liver microenvironment is highly complex and involves many genes whose relevant functions in hematopoiesis are currently unknown.[53]

Similarly, the characterization and comparison of hematopoietic supportive stromal cell lines from the various microenvironments within the conceptus (yolk sac, aorta-mesenchyme, urogenital ridges, gut, liver) and from hematopoietic deficient mouse strains should provide information on induction and growth requirements for the earliest emerging hematopoietic progenitors and stem cells in the mouse embryo. Several laboratories have attempted to examine the role of the microenvironment in the embryonic sites of hematopoiesis (YS and AGM) and have generated stromal cell lines. Yoder and colleagues isolated mesodermal and endodermal cell lines from the YS and showed that they efficiently support the proliferation and differentiation of bone marrow hematopoietic progenitor cells.[54] Another YS-derived cell line that expresses CD34 was shown to support the growth and differentiation of YS hematopoietic progenitors.[55] Two mid-gestation AGM stromal cell lines, AGM-S3 and DAS104-4, have been generated and examined. AGM-S3 cells express VCAM, Sca-1 and CD13 and support hematopoietic progenitors and CFU-S12 from BM Lin^- c-kit^+ $Sca\text{-}1^+$ sorted cells and human cord-blood HSC.[56] The DAS104-4 AGM (endothelial) stromal line isolated on the basis of CD34 expression, efficiently expands fetal liver HSC and maintains their repopulating activity in culture.[57]

Our laboratory isolated and characterized a large panel of stromal clones (about 100) from E11 AGM subregions (aorta mesenchyme (AM) and urogenital ridges (UG)) and the gastrointestinal region and embryonic liver (EL)[58,59] to study more extensively the role of the microenvironment in the AGM region where the first definitive HSC emerge. Our transplantation studies and *in vitro* colony assays using purified HSC from the adult BM and AGM region reveal that some of these AGM stromal clones strongly support HSC and hematopoietic progenitors.[58,59] Several clones exhibit a more potent supportive activity than well-known bone marrow stromal cell lines. Interestingly, the comparison of the supporting activity of stromal clones derived from the AGM and the embryonic liver clearly shows that the AGM microenvironment is the best supporter of HSC in the E11 embryo.[58] Indeed, we found only one EL clone out of 17 (6%) able to support LTC-IC, whereas 9 clones out of 22 (41%) from the UG efficiently supported LTC-IC. Within the AGM region, we identified supportive stromal clones from both subregions, AM and UG. Because HSC are known to emerge from the dorsal aorta, the identification of supportive stromal cells from the AM was not surprising. Importantly, one of our best stromal clones, UG26.1B6, was generated from E11 urogenital ridges suggesting that this may be an important microenvironment in the regulation of HSC *in vivo*, especially when taken together with the findings that the UG contain HSC activity at E12 or after culture of E11 UG explants.[6] We recently observed that direct contact between hematopoietic cells obtained from embryonic tissues and the embryonic stromal cell lines is required to maintain HSC activity in culture.[60] In addition, we have found that UG stromal cell lines support AM-derived HSC better than HSC from other embryonic tissues. Thus, these data indicate that cell-to-cell contacts are required for the support of embryo-derived HSC and that there is a preferred anatomical compatibility between HSC and stromal cells isolated from the spatially/temporally distinct hematopoietic supportive microenvironments.

Phenotypic characterization places the embryo-derived stromal clones along the vascular smooth muscle cell (VSMC) differentiation pathway.[61] Although located along the VSMC hierarchy, recent studies in our laboratory reveal that some UG and AM stromal cell lines are still able to differentiate into other mesenchymal cell types (osteocytes, adipocytes, endothelial cells) when they are cultured under appropriate conditions (*Durand et al., manuscript submitted for publication*). UG stromal cells differentiate into osteocytes and adipocytes, whereas AM stromal cells have a high capacity to form vascular-like tubules and start to express the Flk1 endothelial marker when cultured in matrigel assays. Hence, the mid-gestation stromal cells possess a certain degree of plasticity along the VSMC pathway[62] and present distinct potentialities that appear to be related to their localized origin within the embryo. The careful characterization of this panel of closely related embryo-derived stromal cells with respect to their phenotype, differentiation potential and HSC-supportive capacity should now allow us to identify which sets of genes participate specifically

in the emergence and expansion of HSC occurring in the AGM.

Human hematopoietic development

Of clear interest for our further understanding of the biology of human HSC and their manipulation in clinical scenarios is whether human hematopoietic development is similar to that observed in other species. Indeed, the studies in mouse embryos showing that there are at least two sites of hematopoietic emergence (YS and PAS/AGM) indicate evolutionary conservation in developmental mechanisms and thus strongly suggests that human hematopoietic development is similar. Several elegant studies[63,64] have been performed to examine the origin of human HSC. As in the mouse, prior to the onset of circulation between the embryo proper and the yolk sac, the intra-embryonic splanchnopleura (from 3-4 weeks in gestation) contains cells with multilineage potential (both lymphoid and myeloid), while yolk sac contains cells with only myeloid potential.[65] Beginning precisely at 27 days of gestation,[31,66] just prior to the establishment of liver hematopoiesis, hematopoietic cell clusters are found adhering to the ventral wall of the dorsal aorta. These cells express surface markers characteristic of hematopoietic progenitors and it is thought that these cells colonize the fetal liver and are thus the founders of the human adult hematopoietic system.[67] Furthermore, the ventral aortic mesenchyme underlying the hematopoietic clusters resembles a hematopoietic stromal layer with a morphological cell polarity.[68] Finally, studies show that at least some of the emerging hematopoietic cells within the human embryo appear to be derived from cells in the vascular walls.[69] To analyze the hematopoietic potential of YS and AGM endothelial cells directly, Oberlin and colleagues purified by cell sorting, on the basis of CD31/CD34 and CD45 expression, endothelial and hematopoietic cells from these tissues. Importantly, endothelial $CD31^+/CD34^+$ $CD45^-$ cells isolated from the AGM and cultured on MS-5 stromal cells gave rise to hematopoietic progeny including myeloid, natural killer and B cells. In contrast, the sorted cells from the YS only generated myeloid and natural killer cells. Together, these results strongly support the existence of a hemogenic potential of the intra-embryonic vascular endothelium. Interestingly, immunohistochemistry studies revealed that BMP4, a member of the TGFβ family, involved in the commitment of mesodermal cells into the hematopoietic lineage, is preferentially expressed in the ventral area of the AGM.[70] These results then suggest that the BMP signaling pathway may also play an important role in regulating the first definitive HSC in the human embryo. Thus, as in the other animal models, the intra-embryonic hematopoietic territory contains more potent hematopoietic activity than does the yolk sac and is more likely to contribute to definitive adult hematopoiesis in man. Ongoing and future studies on the intra-embryonic PAS/AGM microenvironment, growth factors and complete genetic program leading to the generation of HSC should provide valuable insight into the manipulation of HSC from adult bone marrow and perhaps the ability to redirect adult endothelium to a hematopoietic fate.

Dissection of the first steps of hematopoiesis using the embryonic stem cell differentiation model

Embryonic stem (ES) cells are pluripotent culture cells that can contribute to basically all tissues after transplantation into a blastocyst embryo. When ES cells are induced to differentiate *in vitro*, they spontaneously form colonies called embryonic bodies (EB). These EB contain precursor cells for many cell types including those for the hematopoietic and neural lineages. Choi and colleagues identified, in 2.5-3.5-day EB cultures, cells able to give rise to blast colonies in response to VEGF. When plated in appropriate conditions, cells from the blast colonies generate both hematopoietic and endothelial cells and are thought to represent the equivalent of hemangioblasts.[71] A more primitive colony, called the transitional colony, has also been identified in 1-day EB cultures. Since the cells from these transitional colonies express an early mesodermal gene (*Brachyury T, personal communication*), in addition to hematopoietic and endothelial genes, they probably represent a more primitive mesodermal cell. Importantly, when replated into methylcellulose, transitional colonies generate precursors for both primitive erythroid and definitive hematopoietic lineages.[72]

Furthermore, comparison of the potential of ES cell lines deficient in some important hematopoietic genes highlights the genetic regulatory cascade of specific transcription factors involved in the development of hematopoietic cells. Keller and co-workers elegantly showed that EB deficient in the *SCL* gene are unable to form blast colonies but still give rise to transitional colonies.[72] Similarly, EB deficient in the *Runx1* gene generate significantly fewer blast colonies than do wild-type EB.[73]

Although, studies on the differentiation of ES cells have contributed enormously to our understanding of hematopoietic commitment and differentiation, the potential of ES cells to give rise to HSC *in vivo* remains unclear. Potocknick and colleagues reported that cells derived from day-15 EB have hematolymphoid potential.[74] In contrast, we showed, that after transplantation into immunodeficient SCID neonates, ES-derived donor cells have only a low and lymphoid-restricted potential.[75] More recently, Kyba *et al.* reported that ES cells transduced with the

homeotic gene, *Hox B4*, acquire the ability to reconstitute the hematopoietic system of irradiated recipients. However, the engraftment appears to be limited to the granulocyte lineage, with the level of lymphoid engraftment in the repopulated mice being very low.[76] Taken together the repopulating potential of ES cells appears so far to be extremely poor as compared to that of HSC derived from various tissues during ontogeny. Development of optimal culture conditions will require further research to reveal all the signals necessary for the emergence of *bona fide* adult transplantable HSC.

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