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Overcoming obstacles in the search for the site of hematopoietic stem cell emergence

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Introduction

Hematopoietic stem cells (HSC) are defined as cells displaying the ability to differentiate into all the various blood cell lineages and to self-renew to sustain hematopoiesis throughout the life of the subject. The first HSCs are generated during embryonic development, with subsequent expansion and then migration through the bloodstream to seed the fetal liver and subsequently, the bone marrow, where blood cells are produced throughout adult life. The stem cell theory of hematopoiesis predicts that all mature hematopoietic elements arise from a HSC via a hierarchical progression of progenitor cell intermediates that display irrevocable changes in the balance between proliferative potential and differentiation. The search for the first HSC to emerge during embryonic development should therefore logically start with the search for the first appearance of blood cells.

The first hematopoietic cells to appear in the developing murine embryo emerge within the extra-embryonic yolk sac (YS) blood islands at embryonic day 7.5 (E7.5) (Moore and Metcalf, 1970).These nucleated erythrocytes express embryonic globins and emerge from distinct progenitor cells in a process called primitive erythropoiesis. The first erythroid progenitors that produce enucleated erythrocytes expressing adult hemoglobin molecules (called definitive erythroid progenitor cells) appear in the YS on E8.25 (Palis et al., 1999). Definitive hematopoietic progenitor cells, including mixed lineage and high proliferative potential colony forming cells, first emerge in the YS, followed by cells in the bloodstream and within the embryo in the paraaortic splanchnopleura region (P-Sp) (Palis et al., 1999). Such a pronounced appearance of blood cells should theoretically herald HSC emergence.

Historical overview of searching for the origin of HSC activity in the embryo

Results from early studies conducted in chick embryos, were indeed consistent with the hypothesis that hematopoiesis and the first HSC, emerge in the YS (Moore and Owen, 1967). However, chick-quail chimera analysis revealed that the contribution of YS to hematopoiesis was only transient and that only intra-embryonic cells were able to provide long-term reconstitution (LTR) (Dieterlen-Lievre, 1975). Similarly, the first cells that can engraft a lethally irradiated adult mouse and provide long term multi-lineage reconstitution have been shown to be present within the intra-embryonic compartment in a region called the Aorta-Gonad-Mesonephros (AGM) of E10.5 embryos (reviewed in Dzierzak and Speck, 2008) with LTR HSC activity emerging slightly later at E11.0-11.5 in the YS, fetal liver and placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). However, when myeloablated newborn pups were used as recipients (because the liver was still an active hematopoietic site and thus, provided an environment consistent with the normal pattern of blood cell migration and organ

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seeding in utero), both YS and P-Sp cells from E9.0 embryos were capable of multi-lineage LTR activity into primary newborn and secondary adult recipient mice (Yoder et al., 1997). These results suggested that repopulating HSC may emerge in more than one site early in development, but are detected somewhat later than the first hematopoietic progenitor cells (a deviation from the stem cell theory of hematopoiesis).

The major limitation in assessing the temporal and spatial emergence of HSC is the fact that LTR HSC activity, tested in either adult or neonatal recipients, is not detectable within the embryo proper, YS, or other hematopoietic tissues until long after the heart has begun to beat and initiate blood cell redistribution throughout the systemic circulation. Obviously, defining the origin of a circulating cell population is nearly impossible using standard reductionist approaches. To circumvent this variable, investigators have adopted several different strategies that include explant culture of tissues isolated prior to initiation of a heartbeat (pre-circulation), use of murine mutants in which no heartbeat develops to examine spatial emergence of hematopoietic progenitor cells, and lineage marking strategies where marking of a putative stem cell precursor occurs prior to systemic blood circulation.

Demonstrating hematopoietic potential: the role of stromal cells

To avoid problems of interpretation induced by the onset of blood cell circulation, several investigators have isolated embryonic tissues pre-circulation and examined hematopoietic cell emergence in vitro. Matsuoka et al reported that precursors for adult repopulating HSCs were present in both YS and P-Sp regions at E8.5, when YS and P-Sp cells were isolated from the pre-circulation embryo and co-cultured in a stromal microenvironment derived from the AGM region (Matsuoka et al., 2001). With only a four day in vitro co-culture period, both YS and P-Sp cells developed the capacity to home and engraft in the bone marrow of adult myeloablated mice. Further work to uncover the cellular and molecular mechanism for this stromal cell effect has not been published. Cumano et al showed that E8.5 P-Sp explants maintained *ex vivo* for two days and then transferred as a cell suspension onto a bone marrow derived stromal cell line were capable of multi-lineage LTR in adult RAG2γc$^{-/-}$ hosts (Cumano et al., 2001). However, cultured YS cells could only provide short-term myelo-erythroid reconstitution and could not repopulate the lymphoid lineages. Thus, determination of the potential of pre-circulation YS and P-Sp tissues for evidence of HSC dependent multilineage differentiation may be dependent upon the unique characteristics of the stromal cells used in the co-culture setting.

A mutant mouse without a heartbeat serves as a novel reagent for hematopoiesis

If one were able to find a mutant mouse in which the embryo developed normally in the absence of circulating blood cells, one could be more assured that the cells isolated in a particular tissue emerged from that tissue. Of course, no embryo can survive without a circulatory system through approximately E11.0 since the organs and tissue become too large for sufficient diffusion of oxygen and nutrients and removal of waste products to sustain embryo survival. *NCX1* is a ubiquitously expressed sodium-calcium exchanger that is expressed only in the heart during embryogenesis and is required for initiation of a heartbeat. *NCX1* gene deficient embryos develop normally until E10.0 but die around E11.5 since the circulation is never established in these embryos (Koushik et al., 2001). Thus, in a recent report, *NCX1* null (*NCX1*$^{-/-}$) embryos provided a window of opportunity to separately analyze the hematopoietic potential of YS and P-Sp cells under a circulation free environment (Lux et al., 2008). The number of primitive erythroid progenitor cells was normal in *NCX1*$^{-/-}$ YS cells compared to WT YS cells, and primitive erythroblasts were found only in the YS, not in the *NCX1*$^{-/-}$ embryo proper as expected since the YS is known to be the only site for primitive erythroblast

emergence. Although, the total number of definitive hematopoietic progenitors within the whole embryonic tissues of *NCX1*$^{-/-}$, *NCX*$^{+/-}$, and WT embryos was not significantly different, essentially all of the definitive hematopoietic progenitor cells were restricted to the vasculature of the *NCX1*$^{-/-}$ YS. Indeed, the YS was engorged with blood cells while the embryo proper was nearly devoid of blood cells. This finding demonstrated a YS origin for essentially all the hematopoietic progenitor cells that seed the fetal liver before E10, as previously predicted (Palis et al., 1999).

The placenta is another hematopoietic niche in which HSC activity is readily detectable around E11.0 and greatly expands over the next 2-3 days (Gekas et al., 2005). Rhodes et. al have examined *de novo* hematopoietic potential of the placenta in the absence of circulation in WT and NCX1 null embryos (Rhodes et al., 2008). In the *NCX1*$^{-/-}$ placenta, CD41^{+} clusters were found in the vessels of the chorioallantoic mesenchyme and developing labyrinth, consistent with *de novo* emergence of hematopoietic cells. Isolation of tissues from E8.5-9.5 *NCX1*$^{-/-}$ and WT embryos and subsequent co-culture with OP9 or OP9-Delta like 1(OP9-DL1) revealed that the placenta, as well as YS and embryo proper (including P-Sp), showed similar potentials for the development of erythro-myeloid and B and T lymphoid cells in WT and *NCX1*$^{-/-}$ embryos. These findings indicate the independent emergence and existence of multipotent hematopoietic progenitors and putative HSC potential in the E9.5 placenta, as well as, the YS and P-Sp. However, direct evidence for the LTR activity of E9.5 *NCX1*$^{-/-}$ YS, P-Sp, and placental cells has not yet been reported using transplantation assays in adult or neonatal hosts. Such assays will be required to determine whether HSCs emerge independently in each hematopoietic site. Additional studies to identify the consequences of a lack of blood flow on the specification of vascular endothelial cells and the impact this plays in the emergence of HSC will also be required.

Developing cell-marking systems to define the origins of hematopoietic cells

In order to directly approach the temporal and spatial origin of blood cells in situ, several cell-tracking or cell-marking systems have been developed which permit cells to emerge in their normal microenvironment and not in a co-cultured situation in vitro. Sugiyama et al generated mouse chimeras by grafting donor green fluorescent protein expressing (GFP^{+}) YS tissue into the YS of host embryos, before circulation commenced, in a whole embryo culture system and traced the donor GFP^{+} YS cells for 66 hours in the host embryos (Sugiyama et al., 2007). GFP^{+} cells circulated into the host embryo and donor YS derived erytho-myeloid and CD19^{+} B cells emerged in the host chimeras, demonstrating the existence of myeloid and some lymphoid potential in the YS before circulation commences.

CD41 is a cell surface integrin enriched in megakaryocyte and platelet cells that is also expressed in hematopoietic progenitors in the murine YS and in HSCs in the AGM region, fetal liver, and adult bone marrow. CD41 promoter driven Cre recombinase mice were generated as a means to identify the HSC and progenitors expressing this integrin *in vivo* when crossed with a reporter mouse strain (Emambokus and Frampton, 2003). Beta-galactosidase (β-gal) detection revealed CD41^{+} cells contributing to embryonic and fetal progenitor cells and even to some extent to the adult HSC fraction. However, the limited expression in adult marrow blood cells suggests that the majority of marrow HSC do not emerge from a CD41^{+} embryonic cell precursor.

The proposal that some endothelial cells give rise to hematopoietic cells (hemogenic endothelium) is suggested based in part upon the observation of hematopoietic cell emergence from the dorsal aorta in murine embryos (Nishikawa et al., 1998). The clusters budding from the aortic endothelium are believed to be HSCs because of the lack of these clusters in the AGM region in Runx1 deficient mutant embryos where HSC activity fails to emerge (North

et al., 2002). Conditional Runx1 deletion in cells expressing TEK/Tie-2, a receptor for angiopoietin-1 expressed in endothelial cells, also resulted in embryonic lethality similar to Runx1 deficient embryos (Li et al., 2006), supporting a role for hemogenic endothelium in putative HSC emergence. However, determination of the spatial origin of the specific endothelium giving rise to HSC remained elusive using such a ubiquitous endothelial promoter.

Samokhvalov et al.(Samokhvalov et al., 2007) developed an advanced cell tracking system and focused on the earliest emergence of labeled cells in the murine embryo. In their system, temporal *Runx1* expression in the embryo was labeled with lacZ or eYFP by single injection of 4-hydroxytamoxifen into pregnant dams (the inactivated allele does not influence embryo survival). Utilizing the restricted Runx1-lacZ expression pattern in the E7.5 YS compartment, those labeled cells that arose and resided in the YS blood islands at E7.5 were subsequently traced throughout embryogenesis and into adulthood via continuous cellular lacZ expression. Thus, $Runx1^{+}$ cells at E7.5 YS were shown to contribute to the endothelium and endothelium associated hematopoietic clusters in the dorsal aorta where HSC activity has been demonstrated to emerge. In addition, erythroid, myeloid, and B and T lymphoid cells in later stages of embryonic development, and much of the HSC fraction in adult BM were identified as labeled YS derivatives. These findings indicate that pre-circulation YS precursors contribute to the long-lived adult marrow HSC compartment. However, these studies are not without some controversy given questions about the half-life and distribution of the injected tamoxifen in the dams, and the sensitivity of staining for rare, potentially labeled cells in the P-Sp and allantoic regions.

In this issue, Zovein et al. have reported that the endothelium lining the ventral wall of the aorta is responsible for HSC emergence in the AGM region and that this hemogenic endothelium is derived from a transient mesenchymal population. To arrive at these conclusions, the authors employed several lineage tracing approaches (Zovein, 2008). A constitutive vascular endothelial-cadherin (VE-cadherin) promoter driven Cre recombinase expressing transgenic mouse line was crossed with the ROSA26R (R26R) reporter transgenic mouse line to mark endothelial cells and their derivatives. Constitutive VE-cadherin Cre×R26R progeny displayed β-gal activity throughout the vasculature of E10.5 embryos, including the endothelium of the ventral wall of the dorsal aorta in the AGM region. At E13.5, progeny from this mating displayed marked cells in 20-40% of the hematopoietic and most of the endothelial cells in the fetal liver, despite VE-cadherin protein expression being restricted largely to endothelial cells (and not hematopoietic cells). The marked progeny were also resident in 20-80% of the adult bone marrow cells (again, far in excess of the reported frequency of cells expressing VE-cadherin protein). These data suggested that the hematopoietic cells were progeny derived from earlier VE-cadherin expressing cells.

Subsequently, the authors chose to move to tamoxifen inducible VE-cadherin Cre × R26R matings to restrict the Cre recombination and cell marking to more specific time points in an attempt to find a point at which AGM and not YS vessels were marked. Surprisingly, induction with tamoxifen administration to the pregnant dams on E9.5 resulted in limited labeling of the vitelline and umbilical arterial endothelium but caused significant labeling of the AGM aortic endothelium and associated hematopoietic cells budding from the aortic endothelium. Labeled endothelial and hematopoietic cells were identified in the fetal liver at E14.5 and in a portion of erythroid, myeloid, T and B lymphoid, and HSC subsets within the bone marrow of mice up to 13 months of age, demonstrating that a temporally induced population of AGM region endothelial cells expressing VE-cadherin produced marked progeny with adult marrow HSC activity. Additional experiments using smooth muscle 22 alpha promoter driven Cre or myocardin promoter driven Cre transgenic mouse lines crossed with the R26R lines demonstrated that early subaortic mesenchyme contributes to AGM aortic endothelium and adult bone marrow cells, while later subaortic mesenchyme fails to label either endothelium

or hematopoietic cells. Thus, only the early and transient AGM mesenchyme population that gives rise to AGM aortic endothelium has the capacity to produce HSC progeny and appears to do so through an endothelial intermediate.

It is apparent that not all adult bone marrow hematopoietic cells were derivatives of a VE-cadherin expressing precursor in the present studies. Is this a reflection of some HSC arising from a non-vascular/non-VE-cadherin$^+$ precursor or a result of the VE-cadherin promoter not functioning optimally in all hemogenic endothelial cells in the various tissues during development? It is also apparent that VE-cadherin$^+$ endothelium of the AGM, placenta, and YS all produced hematopoietic cells at E10.5-12.5, long after the commencement of the systemic circulation. Thus, further studies using inducible tissue specific (AGM, placenta, YS) hemogenic endothelial restricted promoter driven Cre transgenic lines may be required to assess the overall contributions of the HSC derived from each of these unique sites to the adult marrow pool. To generate such reagents, further work will be required to prospectively isolate the hemogenic endothelium from each site to delineate the unique features that distinguish these cells from the other endothelial cells present in these tissues. Once tissue specific hemogenic endothelial cells are isolated, investigators will be able to more thoroughly investigate the genetic regulatory networks that permit hematopoietic cells to emerge from the hemogenic endothelium. Such information may be of great interest to investigators attempting to differentiate HSC from embryonic stem cells or induced pluripotent stem cells, where induction of certain smooth muscle 22 alpha expressing mesoderm subsets and hemogenic endothelium may be the first requirements in seeking differentiation pathways converging to HSC emergence.

Acknowledgments

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