

# Generation of HSCs in the embryo and assays to detect them

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**The precise temporal and spatial emergence of hematopoietic stem cells (HSC) in the murine embryo has been somewhat controversial largely due to differences in the assays utilized to demonstrate HSC repopulating ability. One strategy is to determine where and when one can first detect HSC that engraft upon transplantation into lethally irradiated adult mice. However, knowing that the primary sites and patterns of hematopoiesis change during ontogeny, an alternative strategy is to select transplantation models where the recipient subjects more closely mirror the stage of development of the donor cells. In this regard, perhaps the most relevant assay to determine the presence of HSC activity in the early embryo is to transplant the donor cells *in utero* into recipient embryos. Other recipient models that may permit engraftment of embryonic cells include the use of submyeloablated or genetically HSC deficient newborn mice. Additional informative strategies have included co-culturing embryonic tissues that appear to lack HSC activity, with stromal cells derived from different developmental sites of hematopoiesis to induce HSC emergence, followed by transplantation as a means to determine which embryonic tissues possess HSC potential. This review will highlight some of the various transplantation assays used to identify HSC from embryonic tissues.**

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Murine HSC have been defined as self-renewing cells with the potential to repopulate the hematopoietic system of a lethally conditioned recipient for a prolonged period (at least 4 months) following transplantation (Orlic and Bodine, 1994). Engraftment of the donor cells is expected to result in restoration of all lineages of blood cells in the recipient animal and transplantation of these repopulating HSC are expected to further repopulate secondary lethally conditioned recipient animals. Evidence that large doses of syngeneic murine marrow cells can lead to long-term multilineage engraftment upon transplantation into nonablated or minimally ablated recipient animals has somewhat broadened considerations for the donor and host interactions

required for engraftment but do not significantly alter the requirements to define cells that display HSC activity (Stewart *et al.*, 1993; Ramshaw *et al.*, 1995; Rao *et al.*, 1997). However, the biology of HSC in the early embryo demonstrates several functional differences from HSC present in an adult animal and raise questions as to the most appropriate assays to identify HSC functions early in ontogeny.

Hematopoiesis is a developmentally regulated and tissue-specific process. The primary sites and patterns of hematopoiesis change throughout murine ontogeny. The first blood cells to emerge in the mouse embryo do so in the extraembryonic yolk sac on embryonic day 7.0 in the form of progenitor cells for primitive erythroblasts and macrophages (Palis and Yoder, 2001). Rapid expansion of the primitive erythroblasts and macrophages is required to fill the nascent yolk sac capillary bed with flowing cells that begin to be propelled by the developing heart tube on E8.25. Multipotent hematopoietic progenitors giving rise to definitive lineages also emerge in the yolk sac at this time (Palis *et al.*, 2001). All of these blood elements appear to enter the blood stream and the developing liver appears to be the site of the yolk sac derived-progenitor cell maturation (Palis *et al.*, 1999). The E8.0 paraaortic splanchnopleure (P-Sp)/aorta-gonad-mesonephros (AGM) is a site for HSC but not progenitor cell emergence and these embryonic derived precursors must also seed the liver to mature (Godin *et al.*, 1999). The fetal liver predominates as the primary site of hematopoiesis from E12.0 through birth, although the spleen and bone marrow become active sites of hematopoiesis prior to birth (E18.0).

When and where do the first repopulating stem cells arise in the murine embryo? This question has been addressed using a variety of assays and not surprisingly, several different interpretations of the temporal and spatial emergence of HSC have been reported. Several in depth reviews have addressed this question recently.

Weissman *et al.* (1978) addressed the question of whether yolk sac blood island cells leave the yolk sac and give rise to adult bone marrow hematopoietic stem cells as well as thymic lymphoid cells using an orthotopic synchronic transplantation system. Donor and recipient mice differing at selected cell surface antigens were selected. Donor yolk sac cells (8–10 days post coitus; dpc) were isolated via enzymatic digestion or passage through a fine stainless steel mesh followed

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by velocity sedimentation to separate the hematopoietic mononuclear cells from the epithelial cells (in both cases 92–98% mononuclear cells of >90% viability). Recipient embryos were injected using micromanipulator-assisted microneedle injection ( $10^4$ – $10^5$  yolk sac cells) through the transilluminated uterine wall of the anesthetized dam and into the yolk sac cavity. The authors noted that 'such chimerism was always at low levels and was not always established'. Nevertheless, yolk sac blood islands cells did appear in the bone marrow, thymus and lymphoid organs of injected recipient embryos. The results supported a hypothesis put forth by Moore and Metcalf that hematopoiesis in the yolk sac gives rise to cells that colonize future sites of hematopoiesis in the embryo. Weissman *et al.* did not determine whether all adult hematopoiesis was derived from yolk sac precursor cells or whether the donor yolk sac cells were derived from endogenous yolk sac precursors or from migratory cells from the embryo proper.

Toles *et al.* (1989) tested the ability of yolk sac or peripheral blood cells from E9.0 embryos to engraft via injection into the placental circulation of congenic stem cell deficient recipient mice. Mice with mutations in the c-Kit receptor (*W* locus) are afflicted with diminished receptor signaling and associated defects in HSC, germ cell and melanocyte proliferation and survival. *In utero* transplantation of donor cells into *W* mutant fetuses had been reported to lead to high-level chimerism in the surviving pups. Pregnant *W* mutant dams (11–15 days of gestation when the liver is the primary site of hematopoiesis in the fetus) were anesthetized, their uterine horns exposed and  $5 \times 10^5$  yolk sac or peripheral blood cells (E9.0) were injected through the uterine wall into the placental circulation. Though many recipient fetuses were lost, 6/163 fetal recipients demonstrated evidence of long-term erythroid lineage engraftment from the donor yolk sac cells (three animals were >70% donor cell chimeric at 2 years of age). Unfortunately, no other lineages were examined for evidence of donor-derived cells. Nonetheless, the prolonged persistence of the erythroid chimerism in the recipient animals indicated that functional HSC are present in the E9.0 yolk sac and persist long-term in recipient *W* mutant mice.

Muller *et al.* (1994) tested the ability of yolk sac or intraembryonic (AGM) cells to engraft in recipient mice. They directly transplanted yolk sac or AGM tissues into lethally irradiated mice and reported that the first site to possess long-term repopulating HSC activity was the AGM region on 10 dpc. These and other studies clearly define a role for HSC development in the yolk sac, AGM and fetal liver after 11 dpc in the murine embryo but suggest that the AGM is the first site of HSC emergence (Kumaravelu *et al.*, 2002). One obvious interpretation of these studies is that HSC activity does not appear in the embryo until day 10 postcoitus and this activity is restricted to the AGM region. An alternative interpretation offered by the authors (given the above results with *in utero* transplantation models) is that lack of evidence of HSC engraftment in the lethally

irradiated adult mice may be a result of inadequate expression of homing molecules on the yolk sac or P-Sp cells for the adult bone marrow microenvironment.

Since the liver continues to serve as an active site of hematopoiesis for several weeks postnatally and the liver normally serves as the site for yolk sac and P-Sp/AGM hematopoietic precursor maturation, we hypothesized that yolk sac cells may engraft in newborn recipient mice (Yoder and Hiatt, 1997). To create a site for donor cell engraftment, we employed the use of the chemotherapeutic agent busulfan to administer to pregnant dams on 18 days postcoitus and a sublethal myeloablative condition was established in all the delivered newborn pups (described in detail in Johnson and Yoder, *in press*). Intravenous or intrahepatic administration of E9.0 yolk sac or P-Sp cells into the submyeloablated newborn recipient pups resulted in long-term multilineage reconstitution of their hematopoietic systems (Yoder *et al.*, 1997). Furthermore, isolation of the bone marrow cells of the primary recipients (>6 months) and transplantation into lethally irradiated secondary recipient adult mice resulted in similar multilineage reconstitution in the secondary recipient animals. While these results demonstrate that HSC are present in the E9.0 yolk sac and P-Sp that can engraft in newborn mice long-term, concern has been raised that these results do not specify whether the HSC in the yolk sac and P-Sp arise *de novo* in these sites or are co-mingled since the systemic circulation of the murine embryo has been stated to originate on day 8.0 postcoitus. Recent analysis of the steady-state density of primitive erythrocytes in the vasculature of developing murine embryo supports the onset of the heartbeat at 5–7 somites but indicates that complete establishment of flow between the embryo and yolk sac is not finalized until E10.0 (McGrath *et al.*, 2003). Of interest, on E9.0 the embryo possessed only 1/10 of the primitive erythrocytes of the yolk sac but was composed of 10 times more total cells, suggesting that systemic circulation of the erythrocytes in the embryo was trivial.

An alternative approach to the analysis of embryonic HSC function in fetal or newborn recipients is to attempt to provide a stromal cell microenvironment that supports maturation and expansion of the embryonic HSC into cells that fully engraft and reconstitute the hematopoietic system of lethally irradiated adult mice. Here again, the choice of stromal cell line apparently plays a significant role in determining whether or not the donor embryonic yolk sac or P-Sp cells acquire the ability to engraft in the adult hosts. Stromal cells derived from early embryonic tissues support expansion and emergence of adult repopulating HSC *in vitro* from yolk sac and P-Sp tissues while stromal cells isolated from adult bone marrow support only the emergence of adult repopulating HSC from the P-Sp/AGM region.

Matsuoka *et al.* (2001) utilized an endothelial-like cell line AGM-S3 (derived from 10.5 dpc AGM tissue) in an *in vitro* co-culture system to examine the ability of 8 dpc yolk sac and P-sp cells to develop into HSC with adult repopulating potential. The AGM-S3 cell line had been demonstrated to support the development of both

murine and human HSC *in vitro* (Xu *et al.*, 1998). While neither the yolk sac nor P-Sp cells possessed HSC activity upon isolation from embryos, a 4-day co-culture with AGM-S3 cells effectively permitted the emergence or maturation of HSC that repopulated myeloid and lymphoid lineages for up to 6 months in lethally irradiated recipient adult mice. These results indicated that precursors capable of becoming or producing definitive HSC appear independently in the yolk sac and P-Sp.

Cumano *et al.* (2001) utilized an irradiated stromal cell line S17 (derived from adult murine marrow) as an *in vitro* co-culture monolayer with explanted 8 dpc (5–10 somite stage of development) yolk sac and P-Sp tissues (no added growth factors) for 4 days. A single cell suspension of the recovered cells was injected intravenously into sublethally irradiated lymphoid deficient *Rag 2<sup>-/-</sup>* or lymphoid and natural killer cell deficient *Rag2 $\gamma$ c<sup>-/-</sup>* hosts. While the yolk sac donor cells contributed to short-term myeloid lineage repopulation, only donor cells derived from the P-Sp contributed long-term to myeloid and lymphoid lineages in the immunodeficient hosts. These results suggest that under these culture conditions, the only cells capable of contributing to definitive HSC development are derived from the

intraembryonic P-Sp region. These results are further supported by recent studies employing ‘markers’ of definitive hematopoiesis that provide compelling evidence for the P-Sp/AGM region as the primary site of development of adult repopulating HSC (de Bruijn *et al.*, 2002; North *et al.*, 2002).

While both the yolk sac and AGM region clearly play a role in HSC production and expansion during the onset of fetal liver hematopoiesis, some differences in interpretation of the temporal and spatial emergence of HSC in the early embryo have ensued and exist in part due to differences in the assays used to measure HSC function. This brief overview of some of the transplantation models used to test the function of embryonic HSC has hopefully highlighted the numerous questions that remain with respect to the mechanisms used by HSC to home to the various hematopoietic sites during murine ontogeny. Does each hematopoietic site secrete diffusible factors that attract the progenitors to that organ or are the signals for organ homing displayed on the endothelial cells of the organ vessels or both? These questions await methods for specific imaging of HSC–endothelial interactions and HSC seeding, proliferation and differentiation within hematopoietic organs during ontogeny.

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