

Introduction: spatial origin of murine hematopoietic stem cells

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Hematopoiesis is the developmentally regulated and tissue-specific process of blood cell production. The predominant anatomic site of hematopoiesis changes several times during murine and human ontogeny. Blood and endothelial cells become morphologically identifiable on embryonic day 7.5 (E7.5) in the developing murine yolk sac blood islands.¹ Upon initiation of blood flow through the systemic circulation, blood island-derived progenitor cells are carried throughout the yolk sac and embryo.^{2,3} The liver becomes colonized with hematopoietic progenitor and stem cells at the 28 somite pair (sp) stage of murine development.^{4,5} By E12 the liver is the predominant site of hematopoiesis. Hematopoietic stem cells (HSCs) in the rodent fetal liver subsequently migrate to the bone marrow and contribute to lifelong hematopoiesis.⁶

For nearly 100 years, hematologists have been challenged by the question of how hematopoiesis is sequentially initiated in different organs during mammalian development. At the turn of the century, morphologic evidence that all blood cells appeared to develop from a common precursor cell was accumulating. The “mother” stem cell was identifiable as a mononuclear cell with a large nucleus, prominent nucleoli and deeply basophilic staining cytoplasm.⁷⁻⁹ This cell was present in every tissue displaying hematopoietic activity, leading Maximow⁸ and Danchakoff⁷ to hypothesize that the stem cell must arise independently in each hematopoietic tissue at a specific time or under specific circumstances. Seventy-five years later, the fetal liver has clearly been established as the site of development for HSCs that seed the bone marrow compartment;^{6,10} however, the temporal and spatial origin of the HSCs that seed the fetal liver remains controversial.^{11,12}

More than 30 years ago, Moore and Owen¹³ hypothesized that the sequential emergence of hematopoietic organs throughout ontogeny required an inflow of circulating HSCs from the bloodstream and that HSCs are first formed in the yolk sac. The results of transplantation experiments in which yolk sac cells were injected into irradiated chick embryos¹⁴ served as the basis for this hypothesis. Follow-up studies in the murine system confirmed that the yolk sac was critical to the establishment of hepatic hematopoiesis. Removal of the fetal liver before the 28 sp stage (E9.5) and grafting of the tissue beneath the kidney capsule of an adult recipient resulted in survival of the fetal hepatic tissue, but no hematopoietic elements were present. But administration of hematopoietic cells into the circulation of the recipient mice bearing the fetal tissue grafts resulted in multilineage engraftment in the implanted fetal liver tissue.^{4,5} These data suggested that hematopoiesis did not arise from precursors endogenous to the fetal liver, but the liver tissue did promote growth and differentiation of circulating precursors that lodged therein. Likewise, E7 embryos dissected free from yolk sac membranes grew and developed normally *in vitro* for 2 days, but no blood cells or hematopoietic colony-forming cells were present in any portion of the embryo, including the liver.¹⁵ These studies supported the theory of organ seeding by blood-borne stem cells and the yolk sac as the source of HSCs.¹³

In contrast, some studies in both avian and murine systems have provided data that refutes the hypothesis of the yolk sac as the site of origin of HSCs. Using chick-quail and chick-chick chimeric models, a number of investigators failed to observe yolk sac cell contributions to adult myelopoiesis or lymphopoiesis (reviewed in Dieterlen-Lievre and Le Douarin¹⁶ and Dieterlen-Lievre et al¹⁷). The results of these avian studies contrasted with those of Moore and Owen,¹⁴ but the experimental methods differed in age of donor embryonic tissue, model (unirradiated chimeric host versus irradiated recipient), and period of analysis after transplantation or chimeric grafting.

Efforts to assay directly for the presence of HSCs in the murine yolk sac via transplantation of these cells into recipient animals have yielded results that support or refute the Moore-Owen hypothesis largely dependent upon the age of the recipient. Weissman et al¹⁸ reported that *in utero* transplantation of E8 yolk sac cells into the yolk sacs of congenic nonablated recipients contributed to lymphomyelopoiesis in surviving mice that grew into adulthood. *In utero* transplantation of E9 yolk sac cells into the placenta of stem-cell deficient congenic fetal mice resulted in long-term reconstitution (more than 5 months) of the erythroid lineage in some surviving animals.¹⁹ Thus HSCs that engraft in embryonic or fetal mice and then contribute to adult hematopoiesis can be observed to be present in the E8-9 murine yolk sac. In contrast, the first HSCs that directly engraft in lethally irradiated adult mice are present at E10 in the aorto-gonad-mesonephros (AGM) region within the embryo.²⁰ Adult-repopulating HSCs cannot be identified in the yolk sac until E11, at which time HSCs are already present in the AGM region, the liver, and vitelline and umbilical vessels.²⁰⁻²³ These results are generally interpreted to infer that the yolk sac is not a site where fetal-liver-seeding (and likewise, adult-marrow-repopulating) HSCs develop.

The inability to demonstrate engraftment of yolk sac cells isolated earlier than E11 in lethally irradiated adult mice may be due to a failure of the yolk sac HSCs to home and engraft in an adult microenvironment. Based on the observation that the liver of the newborn mouse continues as an active hematopoietic organ for 1 to 2 weeks following birth,²⁴⁻²⁶ sublethally myeloablated newborn mice were transplanted with E9 or E10 yolk sac cells. In multiple experiments, yolk sac cells were noted to give rise to all lineages of blood cells long-term (more than 11 months) and that marrow from the primary E9 and E10 yolk sac-engrafted recipients also reconstituted B- and T- lymphocyte, granulocyte, and erythroid lineages in secondary lethally irradiated adult recipient mice.²⁷⁻²⁹ These results suggested that yolk sac HSCs capable of repopulating lymphoid and myeloid lineages in adult animals were present prior to E11 but were not identifiable upon direct transplantation into adult recipients. What then is the relationship between yolk sac HSCs that engraft in newborn recipients and contribute to long-lived multilineage repopulation and HSCs from the AGM region, the liver, and vitelline and umbilical cord vessels that directly repopulate all lineages of a lethally irradiated adult mouse?

In this issue, Matsuoka et al³⁰ present novel data that early (day 8.0 and 8.5 post coitus [pc]) yolk sacs (YSs) and para-aortic splanchnopleures (P-Sps) possess HSCs that acquire the capacity to engraft directly and repopulate multiple hematopoietic lineages in lethally irradiated adult recipient mice only after a period of coculture with a stromal cell line previously isolated from the AGM region. AGM-S3 cells are a clonal endothelial cell line derived from the AGM region of a day 10.5 pc murine embryo. These cells have previously been demonstrated to support primitive murine and human hematopoiesis *in vitro*.³¹ In the present work, YSs and P-Sps were isolated from Ly-5.2–staged embryos (stages not stated), 2 embryo equivalents of cells cocultured with the AGM-S3 cells, and were transplanted (with Ly-5.1–unfractionated adult marrow competitors) into lethally irradiated Ly-5.1 adult mice. Both day 8.0 and 8.5 YS and P-Sp cells engrafted and repopulated erythroid, myeloid, and lymphoid lineages but only if cocultured for a minimum of 4 days on the AGM-S3 cells. As one would predict, AGM-S3 cells alone did not repopulate hematopoiesis in the recipients.

These data are significant in addressing several questions regarding the origin of HSCs during embryogenesis. AGM-S3 cells supported the generation of HSCs and spleen colony-forming unit cells (CFU-Ss) but not definitive colony-forming cells (CFCs). While both YS and P-Sp cells produced some CFCs during the first few days of coculture with the AGM-S3 cells, the CFCs formed consisted of some macrophage colonies and erythroid colonies with cells expressing both embryonic and adult hemoglobins reflective of primitive erythropoiesis. In fact, most CFC activity declined in the cocultures at the time that the CFU-S and HSC activity appeared. Thus AGM-S3 cells provide factors that promote generation of CFU-Ss and HSCs but not CFCs. This result is consistent with observations in normal embryos that, while the AGM region does not support the differentiation of mature blood cells, it normally supports the generation of HSCs and some committed definitive progenitor cells.^{32,33}

The finding that CFU-Ss and HSCs simultaneously emerge from the cocultured day 8.0 pc YS and P-Sp tissue is intriguing. The stem cell theory of hematopoiesis purports that the full hierarchy of hematopoietic progenitor and mature cells arises from a single stem cell. Are the day 8.0 pc yolk sac– and P-Sp–derived CFU-S descendants of the HSCs present in these tissues? Several studies have determined that multipotent and committed progenitor cells can be directly assayed from YSs and P-Sps at the E8.25 stage (1–8 sp) of development.^{2,3,34,35} Are these hematopoietic progenitor cells also progeny of the CFU-Ss and HSCs identified in the cocultured day 8.0 pc YS and P-Sp? If so, the cell cycle kinetics and differentiation programs must be vastly accelerated compared to the profile of murine HSC commitment and differentiation observed in the fetal liver and adult marrow microenvironments. An alternative proposal suggests that the embryonic hematopoietic hierarchy emerges in the reverse orientation to the classical adult pathway.³⁶ The hypothesis states the order of appearance of embryonic hematopoietic cells begins with CFCs, CFU-Ss, and finally HSCs, and these cells may have a direct lineage relationship or be derived independently.³⁶ Isolation of the cells displaying the CFU-S and HSC activity in the day 8.0 pc yolk sac and P-Sp and use of stepwise addition of hematopoietic growth factors and/or other stromal elements to the AGM-S3 cocultures may permit examination of the complete hematopoietic hierarchy *in vitro*.

An important consideration in the design of the work of Matsuoka et al was to isolate YS and P-Sp cells from embryos at a time prior to establishment of the systemic circulation. The exact

time of onset of the circulation has never been definitively established. Cumano et al³⁷ and Kaufman³⁸ state that the vascular connection between yolk sac and embryo is established at the 8–10 sp stage. Rugh³⁹ notes that the heart is beating regularly and the circulation is playing a major role in development by the 13 sp stage (day 9 pc). Therefore, while it is not absolutely clear when forward flow of blood through the yolk sac and embryonic vasculature is accomplished, isolation of the day 8.0 pc YS and P-Sp tissue to examine for endogenous HSC emergence is consistent with our current level of understanding that these tissues were not exchanging cells via the circulation.

The simultaneous autonomous emergence of HSC activity in day 8.0 pc YSs and P-Sps is of great interest. Blood cells in the yolk sac differentiate from extraembryonic mesoderm derived from the posterior of the primitive streak.⁴⁰ Blood cells in the P-Sp are derived from the splanchnopleure comprising ventral lateral plate mesoderm apposed to endoderm.⁴¹ What are the specific inductive events that commit mesoderm in 2 vastly different tissue microenvironments to simultaneously produce cells with HSC activity? Many of the key molecules involved in the induction of the blood cell program have been identified (reviewed in Zon,⁴² Shivdasani and Orkin,⁴³ and Belausoff et al⁴⁴). Interpretation of the present data that the AGM-S3 cells somehow “educate” existing embryonic HSCs to display competence to engraft in the adult conditioned recipient presents a new complex area for investigation. Of interest, Cai et al⁴⁵ recently reported that the level of expression of the Runx 1 transcription factor influences the temporal and spatial appearance of adult repopulating HSCs. Expression of the $\beta 1$ integrin receptor is necessary for P-Sp cells to home and engraft in fetal liver and the adult marrow cavity.⁴⁶ These 2 examples suggest that a wide variety of changes in cell function may be occurring in the YS and P-Sp cells in the presence of the AGM-S3 cells.

While day 8.0 pc YS and P-Sp cells have the capacity to engraft and repopulate the hematopoietic system in conditioned adult hosts if “educated” by AGM-S3 cells, one may question whether either site normally contributes the HSCs that seed the fetal liver and establish the HSCs for lifelong hematopoiesis. That question remains to be addressed. Additional questions include the following: (1) What is the frequency of these HSCs in the day 8.0 pc YS and P-Sp and are there differences in repopulating ability between HSCs emerging in the yolk sac and those in the P-Sp? (2) What is the relationship between the HSCs generated *in vitro* in coculture with the AGM-S3 cells and the embryonic HSCs that repopulate conditioned newborn recipients? (3) Are there other tissues, such as the fetal liver, that may comprise endothelial cells that also possess the capacity to induce adult engrafting properties from day 8.0 pc YSs and P-Sps?

Perhaps the most significant outcome of the studies of Matsuoka et al is the demonstration of murine HSC induction *ex vivo*. At present, it is unclear if the YS and P-Sp cells that engrafted the conditioned adult recipients were cells already committed to a hematopoietic fate (an embryonic HSC) or if these HSCs generated *in vitro* were derived from a more primitive cell progenitor (hemangioblast or even residual mesoderm) or another cell type (endothelium). The increasing awareness that HSCs display a high degree of plasticity provides new challenges to define experimental conditions that will permit stem cell induction and commitment to specific cellular lineages. The model presented by Matsuoka et al suggest that HSCs may be modified by microenvironmental manipulation to display new properties and functions but with retained *in vivo* multipotency.

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