

Hematopoiesis from embryonic stem cells: Lessons from and for ontogeny

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Cellular therapies derived from embryonic stem (ES) cells have acquired new interest and urgency with the demonstration that embryonic stem cells can be established from human blastocyst-stage embryos. Our ability to derive therapeutic cells from differentiating ES cell cultures will ultimately depend on our understanding of the embryonic developmental processes that direct the differentiation of pluripotent cells into transplantable lineage-specific stem cells, and on our ability to recapitulate these processes in vitro. In this review, we evaluate the work that has been done to date on the hematopoietic differentiation of ES cells, and discuss this in the context of what is known about the embryonic origin of the hematopoietic stem cell. © 2003 International Society for Experimental Hematology. Published by Elsevier Inc.

Why use ES cells?

The amazing developmental potency of embryonic stem (ES) cells is illustrated by their ability to chimerize any tissue of a developing embryo, including the germ line itself. While this potency has been widely exploited for transgenesis, it also has the potential to provide the researcher and clinician with a powerful tool for the study and therapeutic application of the primitive cells that constitute the origin of each distinct developmental lineage. With respect to the hematopoietic lineage, the primary scientific advantage of ES cells over the embryo relates to the accessibility of cellular material for study. The hematopoietic lineage is one of the earliest to arise in mammalian development, being specified from the first set of mesodermal precursors generated by gastrulation. The tiny size of the murine embryo and the limited number of hematopoietic founder cells at this stage make many types of biochemical, molecular, and cellular analyses unfeasible. On the other hand, ES cells grown in quantity and differentiated synchronously can provide large amounts of cellular material for analysis.

In the clinical setting, ES cell-derived hematopoietic stem cells (HSCs) would have a number of advantages over HSCs from the conventional sources of bone marrow and umbilical cord blood. Suitable donor bone marrow is often in short

supply, and cord blood, although bankable, contains a much lower absolute number of HSCs, making it less than ideal for the treatment of adults. Both sources of hematopoietic tissue suffer from our inability to reliably expand the long-term repopulating HSC in vitro. In contrast, ES cells can be expanded indefinitely in vitro and may eventually be derived by reprogramming of somatic cells or taken from a bank representing major haplotype combinations. This unlimited expansion not only allows large absolute numbers of HSC to be generated, but also enables the replenishment of banked ES samples. Another advantage of an ES-derived HSC would be its immaturity compared to the bone marrow-derived HSC, an advantage shared by cord blood. Aged bone marrow accumulates defective elements [1] and is clinically associated with lower long-term survival and greater incidence of graft-vs-host disease [2]. An ES-derived HSC would in principle be juvenile, and not suffer from the adverse effects of aging. ES-derived HSCs would share in several other advantages of cord blood over bone marrow transplantation: low risk of pathogen contamination, lower risk of graft-vs-host disease, and collection at no risk to the donor [3]. In the event of poor engraftment, supplemental doses of HSCs from the same ES donor genotype could routinely be administered. Finally, in the era of tissue therapy an important role for the ES-derived HSC may be the ability to induce hematopoietic chimerism in individuals undergoing

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transplants with other types of ES-derived tissues. For example, a diabetic patient undergoing a transplant with ES-derived insulin-producing islet cells would need lifelong immunosuppression if the ES cells were anything other than isogenic. However, it is well established that cotransplantation of hematopoietic stem cells along with non-hematopoietic donor tissue can induce tolerance to the nonhematopoietic graft [4–7]. HSC transplants may also be able to block preexisting autoimmunity [8]. The ability to generate engraftable HSCs isogenic to the actual therapeutic cells would thus enable immunologic tolerance in the absence of immunosuppression. Since even low levels of chimerism are tolerizing, nonmyeloablative conditioning might be sufficient. This effect would enable a large patient population to access cellular therapies from a limited bank of approved ES cell lines.

Hematopoietic stem cell ontogeny

Being derived from the inner cell mass of the blastocyst embryo, ES cells are poised to recapitulate very early embryonic events. It is therefore worth considering hematopoiesis in this context. The hematopoietic needs of the embryo are distinct from those of the adult, and the process of hematopoiesis has evolved temporal and spatial distinctions to address these needs as development progresses. The principal distinctions relate to oxygen transport and adaptive immunity. The placenta provides an immunologically privileged environment and necessitates the extraction of oxygen from the maternal blood. The early embryo lacks lymphocyte production. Instead, the earliest mode of hematopoiesis is specialized for the production of primitive (i.e., embryonic) erythrocytes, which express isoforms of hemoglobin with higher oxygen affinity than the hemoglobin of definitive (i.e., adult) erythrocytes [9,10]. This primitive mode of hematopoiesis takes place in the yolk sac concurrent with vasculogenesis beginning on the seventh day of gestation. What begin as thickenings in the yolk sac inner mesodermal layer develop into blood islands: endothelial sacs containing hematopoietic cytotoblasts and differentiated primitive erythrocytes. The spatial and temporal association of hematopoiesis and vasculogenesis led His to propose a common origin of these lineages over a century ago [11]. By 11 days of gestation in the mouse and about 6 weeks in the human, the fetal liver becomes hematopoietic and remains the dominant site of hematopoiesis until the bone marrow takes over this role. Early experiments in mouse embryos documented that hematopoietic progenitor activity exists in the yolk sac beginning on day 8 of gestation and does not appear in the embryo body until day 10, a time point after the onset of circulation [12]. In this work, embryonic bodies separated from their yolk sacs at day 7 and cultured *in vitro* did not acquire progenitor activity, whereas their corresponding yolk sacs did. Therefore it was proposed that the yolk sac was the site of *de novo* generation of hematopoietic stem cells. The

hematopoietic stem cell pool was proposed to originate in the yolk sac, then migrate initially to the fetal liver and finally to the bone marrow, where it would reside for the remainder of adult life (Fig. 1A) [13].

This view of murine hematopoiesis contrasts with observations from other vertebrate model systems in which a second, intraembryonic wave of *de novo* hematopoiesis takes place. This was first demonstrated in the amphibian, *Rana pipiens*, with the transplantation of cytogenetically marked dorsal mesodermal tissue [14,15]. These transplants, which were distinguished by identifying the difference in DNA content between diploid and triploid cells, were shown to differentiate into circulating erythrocytes. A similar intraembryonic hemogenic site was demonstrated for avian embryos through interspecies grafts between chick and quail, in which the yolk sac was derived from one species and the embryonic body from the other [16]. In these chimeric embryos, colonization of hematopoietic sites and development of definitive erythropoiesis was observed to derive from cells of the embryonic body.

A corresponding intraembryonic hemogenic site was eventually discovered in the mammalian embryo [17,18]. This site, referred to as the AGM (for aorta-gonad-mesonephros), comprises splanchnopleural mesoderm from the

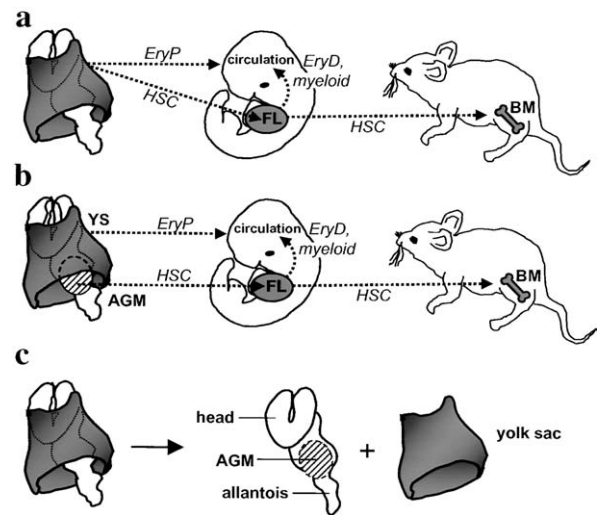


Figure 1. Alternative models for the origin of the definitive HSC. (A): Yolk sac origin. HSCs were initially proposed to arise in the yolk sac (gray) where they contributed primitive erythrocytes to the embryonic circulation. They then migrate to the fetal liver (FL) where they switch to producing definitive erythroid and myeloid progenitors, and finally to the bone marrow where they provide for the life-long hematopoietic needs of the adult. (B): AGM origin. With the discovery of an intraembryonic hemogenic site, the model has been modified to allow for two independent sites of HSC generation. According to the current model, the primitive HSC of yolk sac contribute only an early wave of primitive erythrocytes to the embryonic circulation before exhausting their potential. The definitive HSC of the AGM exclusively populate the fetal liver, providing fetal hematopoiesis, and then transit to the bone marrow to provide life-long adult hematopoiesis. (C): Dissection of the presomite murine embryo showing the location of the AGM and yolk sac in A and B above.

lower trunk and caudal region of the embryo, and corresponds anatomically to the blood-forming dorsal lateral plate mesoderm in amphibian embryos. When embryonic tissues were assayed for hematopoietic potential by injection into lethally irradiated adult mice, the AGM at day 10 was the earliest tissue to demonstrate adult engraftment potential (although in only 3 of 96 mice) [19]. By day 11 both the yolk sac and AGM have repopulation potential (seen in 10 of 17 and 11 of 19 recipient mice, respectively). But since embryonic blood circulation is already established by day 10, it was difficult to assess the site of origin of these HSCs. In an experiment that suggested the autonomous generation of HSCs in the AGM, the yolk sac and AGM were dissected from day-10 embryos and cultured separately *in vitro* [20]. After two days of culture, only the AGM generated adult-engraftable cells. Using a similar approach, but with precirculation embryos, a second group showed that lymphoid cells could be derived *in vitro* from explanted and cultured AGMs from 2 to 8 somite pair embryos but not from their corresponding yolk sacs [21]. These experiments, particularly the inability of *ex vivo*-cultured yolk sac to generate lymphoid progeny or to engraft in adults, prompted a reevaluation of murine embryonic hematopoiesis along the lines established for avian and amphibian model systems. It is now accepted wisdom that definitive HSCs, those that function in definitive hematopoiesis and persist throughout adult life, arise in the AGM. The yolk sac HSCs are relegated to providing a transient burst of primitive erythropoiesis and are believed incapable of the two major hallmarks of definitive hematopoiesis: lymphoid differentiation and repopulation of lethally irradiated adult recipients (Fig. 1B) [22].

Hematopoiesis from ES cells: primitive or definitive?

The ability of ES cells to generate blood *in vitro* was initially noted by Doetschman et al. [23]. These investigators differentiated ES cells in suspension culture and noted that the cystic multilayered structures that arose (embryoid bodies; EBs) were superficially similar to the egg-cylinder-stage embryo, and that many of these EBs developed overtly hemoglobinized blood islands. The capacity of EBs to generate primitive erythrocyte colony-forming cells (EryP-CFCs), as well as the time course of CFC production, suggests that EB hematopoiesis recapitulates the primitive hematopoiesis of yolk sac [24–26]. The inability of yolk sac HSCs to contribute to the definitive HSC pool would thus have significant negative implications for the use of ES cells as a source of HSC for hematopoietic transplantation, at least with established methods of differentiation.

Since the first demonstration of blood differentiation in EBs, many groups have attempted to transplant EB-derived cells into conditioned adult recipients. Although the failure of EB-derived cells to generate spleen colonies (CFU-S) has been documented [24], the many failed attempts to generate

adult hematopoietic chimeras for the most part have not been published. In the two decades that have passed since the initial observation of blood differentiation from ES cells, there have been a few sporadic reports of success. One study documented ES-derived hematopoietic contribution detected by male-specific polymerase chain reaction (PCR) [27]. In this study, EBs were differentiated prior to transplant for two days in the presence of LIF (a cytokine that inhibits ES cell differentiation) and 4 days in its absence. Because the male cells that were detected in these mice were not rigorously characterized, it is possible that these cells were not actually hematopoietic. The injected population was differentiated for only 4 days in the absence of LIF and likely retained significant numbers of pluripotent cells with tumorigenic potential, which might have contributed to circulating cell populations. Another study documented long-term lymphoid contribution in lymphoid-deficient Rag-1^{-/-} mice with CD45⁺, AA4.1⁺, B220⁻ cells from day-15 EBs [28]. This successful experiment contrasts with a similar experiment in which Percoll-fractionated EB cells were transplanted into immunodeficient SCID neonates [29]. This latter report documented only a very low, and lymphoid-restricted, contribution of the ES-derived donor cells, which was most reproducible with cells from EBs differentiated for 11 to 13 days. It is notable that both of these examples of engraftment made use of EBs differentiated for many days past the time point at which the hematopoietic lineage is initially specified. While hematopoiesis in early-differentiated EBs reproducibly recapitulates yolk sac hematopoiesis, it may be the case that in EBs left to differentiate for longer periods, a cell population resembling AGM splanchnopleural mesoderm is occasionally generated. Within the embryo, this tissue arises as part of a highly organized structure, and its specification within the relatively disorganized structure of an EB, if it happens at all, can only be haphazard and incomplete. The failure to reproducibly generate such cells *in vitro* thus reflects the chaotic nature and intrinsic variability of the *in vitro* differentiation process, a variability that is amplified over lengthy periods of cell culture. Another source of variability is the use of nonstandardized components. One can imagine that fetal calf serum, a ubiquitous component in the examples cited above and notorious for batch-to-batch variation, may occasionally contain high levels of a factor that induces AGM-type hematopoietic differentiation. Conditioned media can also be highly variable. In the SCID neonate reconstitution experiment described above, EBs were grown in the presence of Pokeweed mitogen spleen-conditioned medium, a veritable witches' brew of cytokines.

As an alternate to differentiation in suspension aggregates, ES cells can be differentiated directly on tissue culture plates, although under this regimen they do not undergo hematopoietic differentiation [30]. However, the inclusion of stromal cells, also a nonstandardized component, can promote efficient hematopoietic differentiation. The ES differentiation literature contains one example of successful adult

hematopoietic engraftment using a combination of coculture on a bone marrow stromal cell line, RP010, and conditioned medium from a fetal liver stromal cell line, FLS4.1 [31]. As these stromal cell lines are not generally available, this result has not been replicated. While most stromal cell lines result in an abundance of macrophage growth, the stromal cell line OP9 [32], derived from bone marrow of the M-CSF-deficient (op/op) mouse, supports lymphoid as well as myeloid differentiation from ES cells *in vitro* [33]. ES cells grown directly on OP9 monolayers differentiate first to nonrefractile mesodermal colonies, and upon trypsinization and replating on fresh OP9, to colonies of semiadherent hematopoietic blast cells. These secondary colonies contain progenitors of lymphoid or myeloid lineages, and in some cases both, implying the presence of an HSC. Although they are clearly capable of lymphoid differentiation, ES cells cultured on OP9 are unable to engraft conditioned adult mice (Toru Nakano, personal communication). If lymphoid differentiation and adult engraftment are the dual hallmarks of definitive hematopoiesis, this result is difficult to explain. It hints at problems with a simple model proposing the independent origin and functional distinction of the stem cells for primitive and definitive hematopoiesis.

Skeletons in the closet

The now widely accepted belief that the HSCs of the murine yolk sac do not contribute to the definitive HSC pool rests principally on the elegant demonstrations, described above, that yolk sac tissue explanted before the onset of circulation and cultured *in vitro* is devoid of lymphoid potential and incapable of engrafting conditioned adult recipients. Bearing in mind that it is difficult to prove a negative proposition with negative data, it is worth considering alternative explanations for these results. In the work of Medvinsky and Dzierzak [20], day-10 explanted AGMs cultured *in vitro* could generate adult-repopulating tissue, while day-9 explants could not. This suggests either that the relevant cells migrate into the AGM between days 9 and 10, or that the *in vitro* culture conditions applied were insufficient to recapitulate the expected hematopoietic development of this tissue. Accepting the latter, it stands to reason that the culture conditions may likewise have been limiting for proper development of the explanted yolk sacs. The same explanation could account for the lack of lymphoid progenitor derivation from explanted yolk sacs documented by Cumano, Dieterlen-Lièvre, and Godin [21]. These investigators cultured yolk sac and AGM tissue for 5 days on the bone marrow stromal cell line, S17 [34], followed by B- or T-lymphoid-specific culture conditions (further growth on stroma, or coculture with embryonic thymic rudiments, respectively). There is no *a priori* reason to expect yolk sac cells to develop appropriately while spending 5 days on a bone marrow stromal cell monolayer. In fact, an elegant *in vitro* culture model based on combining explanted, alymphoid thymic rudiments

with cells from other hematopoietic tissues had successfully derived lymphoid cells from precirculation (day-8) yolk sacs many years earlier [35]. In contrast to the results of Cumano and colleagues, when this technique was used to compare the lymphoid potential of precirculation (2–9 somite) yolk sac against corresponding embryonic body cells, the yolk sac and not the embryonic body generated lymphoid progeny [36]. This method appears to model the natural course of embryonic thymocyte development by immediately bringing together the appropriate cells with the appropriate microenvironment, whereas the method of Cumano and colleagues adds 5 intervening days in a developmentally inappropriate microenvironment (alone in tissue culture and/or culture on S17 bone marrow stromal cells [21,37]). Adding to the confusion, S17 stromal cells have recently been shown to induce the hematopoietic differentiation of pluripotent human ES cells, with early cobblestone-like colonies initiating within as few as 5 days of coculture [38]. With such a dramatic effect on pluripotent cells, S17 stroma are unlikely to provide a neutral environment for AGM culture. Intriguingly, between day 8 and day 8.5 another pluripotent cell population, the primordial germ cells, have begun their migration through the AGM on their way towards the genital ridges [39]. Since primordial germ cells, like ES cells, have been reported to undergo hematopoietic differentiation *in vitro* [40], it is not out of the question that they may be induced by S17 coculture to undergo lymphoid differentiation. At later time points (30–40 somites) when the AGM can be dissected into its constituent components, lymphoid potential in this assay is actually present in the gonad, although it is most enriched in the aorta [41].

Recently, experiments similar to those of Cumano et al. have been undertaken using human embryos [42]. In this work, yolk sacs and AGMs were cultured for 2 days intact and then disaggregated on MS-5 stromal cells [43], and cultures were assayed for lymphoid and myeloid differentiation. At the earliest precirculation time point studied, day 19, both yolk sac and AGM were capable of lymphoid and myeloid differentiation, although their lymphoid potentials were restricted in both cases to the NK cell lineage. The MS-5 system supported self-renewal of AGM cells considerably better than those of yolk sac, as CD34⁺ cells were nearly exhausted from yolk sac cultures after two weeks. B lymphocytes arose in the third week of culture, however only from embryos older than 24 days, and then only from the AGM with an efficiency of about 70%. The authors assayed for T lymphocytes by culturing AGMs and yolk sacs intact for 10 days, followed by repopulation of NOD-SCID fetal thymuses, and this potential was only seen in the AGM after day 26, at an efficiency of 56%. While the lack of derivation of all three classes of lymphocyte from yolk sac could be interpreted to mean that yolk sac does not contribute to the definitive HSC pool, it could also be attributed to suboptimal conditions for the expansion of the yolk sac HSCs. The fact that precirculation AGM and yolk sac are similarly

restricted to myeloid and NK cell differentiation, and that B- and T-lymphocyte differentiation potential is acquired at a later stage in the AGM, suggests that developmental maturation of the HSC is necessary, even for those HSCs arising in the AGM.

The current model for the origin of the adult HSC pool is also problematic in its reliance on an assay for adult behavior (reconstitution of conditioned adults) to characterize an embryonic cell type. There is no reason that the embryonic progenitors should be able to home to bone marrow when they arise before bone marrow exists. Reasoning that the homing behavior of the HSC must change with the course of development, being first focused on the fetal liver, Yoder and colleagues have shown that day-9 and day-10 yolk sac tissue, although unable to repopulate adults, is in fact able to engraft and sustain long-term lymphoid-myeloid hematopoiesis when injected into the livers of conditioned newborns [44,45]. These experiments are reminiscent of earlier work which had shown that trans-placental injection of yolk sac tissue from as early as day 8.5 into recipient embryos could result in eventual donor contribution to definitive hematopoiesis [46,47]. To explain these results, the current model would require that repopulating cells of the day-9 yolk sac were transported there from the AGM through the circulation [48]. The onset of circulation had been assumed to be at embryonic day 8.5, between the 8- and 9-somite-pair stage [21], and perhaps as early as the 5-somite-pair stage [37]. However, recent work has demonstrated that while the first heartbeat can be detected by 5 somites, and some vascular flow by 7 somites [49], circulation of erythrocytes increases only incrementally following the 8-somite stage as vascular remodeling progresses. A steady-state erythrocyte density, indicating free flow of cells between the yolk sac and embryo proper, is not achieved until embryonic day 10 [50]. These data reduce the likelihood that the embryo- and neonate-repopulating cells of the day-9 yolk sac originate in any tissue other than the yolk sac itself.

Recently, several groups have undertaken a painstaking analysis of the origin of colony-forming cells and adult-repopulating cells in embryonic development. In the first study, the yolk sac was found to contain, in addition to all primitive erythroid CFCs, all of the precirculation embryo's definitive erythroid and myeloid CFC content, indicating that the yolk sac plays a much larger hematopoietic role than simply contributing primitive erythrocytes to the embryonic circulation [26]. In the second study, the earliest multipotent myeloid progenitor that can be grown without stromal support, the HPP-CFC, was also found to be yolk sac-restricted in the precirculation embryo. This progenitor was found in the blood on day 9 but did not appear in the AGM until day 9.5 (and there at levels 10-fold lower than in yolk sac), again suggesting a yolk sac origin for this CFC [51]. In the third study, limiting-dilution analysis was used to measure the number of adult-repopulating units (RUs) produced by various embryonic tissues [52]. This study found that both

yolk sac and AGM had on average 1 RU at day 11, about 2 on day 12, and less than 1 by day 13, while the circulation contained numbers increasing from 1 on day 11 to about 6 RUs by day 13. The liver, in contrast, increased from 0 on day 11 to over 250 by day 13. By comparing the RU content of in vitro-cultured day-11 and day-12 yolk sac and AGM explants, the authors speculate that the liver acquires RUs from the circulation in two waves: the first from the AGM between days 10 and 12, and the second from the yolk sac between days 11 and 13. Since the 100-fold increase in total embryonic RUs between days 11 and 13 occurs too rapidly to be explained by cell proliferation, the authors speculate that the RUs deriving from both the yolk sac and the AGM originate in these tissues in an immature state, and acquire the ability to repopulate adults with developmental maturation associated with fetal liver homing.

The concept that the yolk sac HSC can be matured to a definitive state has been bolstered by two recent complementary experiments. In the first, precirculation yolk sac cells were reprogrammed extrinsically by several days' exposure to an AGM-derived stromal cell line, and injected into conditioned adult mice [53]. They were then able to engraft and contribute to long-term lymphoid-myeloid hematopoiesis. The second experiment used a cell-intrinsic approach: retroviral transduction of *HoxB4*, a transcription factor selectively expressed in the definitive HSC [54] (see also below). After expansion on OP9 stroma, *HoxB4*-expressing yolk sac cells acquired a similar definitive hematopoietic potential.

Considering three and a half decades of experimental data together, both before and since the reevaluation caused by the AGM explant results of Cumano, Medvinsky, and colleagues, the precise relationship of yolk sac progenitors to the definitive HSC pool remains incompletely understood. A reasonable working hypothesis would be that the murine yolk sac autonomously provides all of primitive hematopoiesis as well as stem cells which, together with a wave of stem cells from the AGM, are capable of maturing to contribute to the definitive HSC pool (Fig. 2).

The principal attraction of the AGM-only model of definitive hematopoiesis has always been its attempt to unify the view of murine hematopoiesis with that of other vertebrates. However, even in other vertebrate systems, the potential of

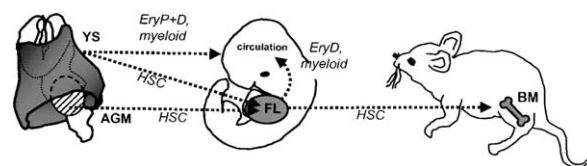


Figure 2. Model of dual contribution of yolk sac and AGM to the definitive HSC pool. The HSCs of yolk sac provide all of the early embryo's primitive erythrocytes, as well as early definitive erythroid and myeloid progenitors. The fetal liver is colonized by these HSCs as well as HSCs arising in the AGM, and together these two pools contribute to fetal hematopoiesis. The HSCs that colonize the bone marrow are thus a heterogeneous population, derived partly from the yolk sac and partly from the AGM.

the yolk sac or homologous tissue to contribute to definitive hematopoiesis is controversial. Although engrafted yolk sacs do not contribute to definitive hematopoiesis in the avian model, yolk sac cells injected into irradiated chick embryos do [55]. In *Xenopus* grafting experiments, stem cells derived from the ventral blood island (homologous to the yolk sac) are long-lived and contribute to adult hematopoiesis for at least two years, although at one fourth the efficiency of dorsal lateral plate (homologous to AGM) stem cells [56]. It should also be borne in mind that one of the evolutionary distinctions between mammals and other vertebrates is the elaboration of enhanced or novel roles for mammalian extra-embryonic tissues. It is not unreasonable to imagine that these evolutionary changes may include a correspondingly enhanced role for extraembryonic HSCs. However, until we know with certainty the relative contributions of murine yolk sac and AGM to definitive hematopoiesis, this possibility is speculative. And this will likely remain the case until methods are developed to genetically mark early yolk sac and AGM tissue selectively *in vivo*, or until embryo culture methods improve to the point of enabling development to day 12, such that grafting experiments might be initiated and analyzed completely *in vitro*.

From the perspective of ES cell differentiation and its preference for the yolk sac mode of hematopoiesis, the question of greater importance is not to what extent does the yolk sac contribute to the definitive HSC pool, but what are the processes that specify the distinction between primitive and definitive HSCs, and how might we recapitulate these processes *in vitro*?

Modeling early hematopoietic events using ES cells

Because hematopoietic CFCs develop within 6 days of EB culture, it is possible to investigate the developmental precursors to the hematopoietic lineage by disrupting the EBs at earlier time points. Making use of colony assays and replating experiments, Keller and colleagues have identified the properties of several cells along the differentiation pathway from pluripotent to hematopoietic. A crowning achievement of this approach was the identification of a cell in day-3 EBs able to give rise to a blast colony in methylcellulose culture in response to vascular endothelial growth factor (VEGF) [57]. By replating cells from these blast colonies into other conditions, it was determined that this colony-forming cell (named the BL-CFC) was capable of differentiating into both primitive and definitive erythroid and myeloid hematopoietic precursors, as well as into endothelial cells [58]. The combination of endothelial with hematopoietic potential thereby identifies the BL-CFC as the long-awaited hemangioblast [11,59,60]. Although the BL-CFC has not yet been derived from embryos, a number of studies since this seminal observation have confirmed the relationship between blood and endothelium *in vivo* [61–65].

A more primitive cell type giving rise to a compact “transitional” colony can be identified one day earlier in EB differentiation [66]. Transitional colonies express the early mesodermal marker *brachyury* (*T*) in addition to hematopoietic and endothelial markers, whereas BL-CFCs express the latter but not *brachyury*. Transitional colonies can be replated to give BL-CFCs, but BL-CFCs can generate neither secondary transitional colonies nor secondary BL-CFCs. The transitional colony-forming cell thus represents an early mesodermal precursor to the hemangioblast.

By performing this type of analysis with ES cell lines carrying various mutations, it has been possible to dissect some aspects of the genetic regulation of hematopoietic commitment. The basic helix-loop-helix transcription factor SCL is expressed in all embryonic hemogenic sites, and is absolutely required for embryonic hematopoiesis [67–69], in addition to being expressed in the embryonic vasculature and required for proper vascular remodeling [70]. Expression of SCL in *Xenopus* embryo animal cap explants induces hematopoiesis [71] and, if ectopically expressed together with its binding partners GATA-1 and LMO2, ventralizes embryos and induces hematopoiesis throughout [72]. SCL-null EBs, which are completely devoid of hematopoiesis, were found also to lack BL-CFCs [66], and to have a corresponding increase in a more compact “blast-like” colony with endothelial but not hematopoietic potential [73]. Although this result suggested that SCL expression was necessary at the level of the hemangioblast to enable hematopoietic differentiation, evidence from zebrafish had placed SCL upstream of the hemangioblast [74]. In this work, injection of SCL mRNA led not only to increased blood formation, but also to increased expression of the endothelial markers Flk-1 and Fli-1, and did so in nonaxial mesoderm at the expense of muscle. This was most consistent with a role for SCL in the restriction of early multipotent mesoderm into hemangiogenic mesoderm.

Flk-1 is a receptor tyrosine kinase that is activated by VEGF [75]. Loss of Flk-1 blocks endothelial development and day-8.5 yolk sac hematopoietic development [76], and Flk-1-null ES cells fail to contribute to hematopoiesis or endothelium in chimeric embryos [77]. These results agree nicely with the requirement for VEGF in the BL-CFC assay. Flk-1-null EBs also have greatly reduced numbers of BL-CFCs; however, they have normal numbers of hematopoietic colonies if allowed to mature to day 9, and they are capable of endothelial development [78]. This result is difficult to reconcile with a simple model postulating that VEGF signaling leads to specification of the hemangioblast. In addition, Flk-1-null embryos have normal numbers of hematopoietic progenitors at day 7.5, suggesting that the Flk-1 defect may relate not to specification of hemangioblasts, but rather to their inability to properly migrate to sites that would allow their survival and proliferation [78].

The confusing relationship between Flk-1 signaling, SCL expression, and hemangioblast development has been the

object of intense recent interest. One approach to disentangle the roles of Flk-1 and SCL has been to mark SCL-expressing cells by knockin replacement of one copy of *SCL* with human *CD4*, which allows cell sorting of single- and double-positive populations at different times during EB development [79]. This demonstrated that Flk-1 is expressed first, that SCL expression occurs in a subpopulation of Flk-1⁺ cells, and that coexpressors can subsequently become single-positive for one or the other marker. BL-CFC were highly enriched in the double-positive population; later hematopoietic progenitors were found in both the double-positive and SCL single-positive populations; and endothelial progenitors were either double-positive or Flk-1 single-positive. Maintenance of SCL expression is thus correlated with hematopoietic commitment and maintenance of Flk-1 expression with endothelial commitment, while both are correlated with hemangioblast generation. Using the OP9 differentiation system, another group has generated a CRE-inducible allele of *SCL* on a null background and studied the time dependence of the requirement for SCL [80]. SCL was only effective in restoring hematopoietic potential in this system if expressed between days 2 and 4 (which is before hematopoietic CFC can be detected) and ineffective after this point. Although Flk-1⁺ cells were generated in the absence of SCL, they were unable to differentiate towards hematopoiesis. A complementary approach taken by a third group has been to examine the hematopoietic and endothelial potential of Flk-1-null ES cells with one allele of *Flk-1* being a knockin replacement with *SCL* [81]. In embryos derived from these ES cells, the knockin allele behaved as a weak suppressor of the hematopoietic and endothelial defects associated with loss of Flk-1. In the BL-CFC assay, these ES cells generated blast colonies at the same frequency as *Flk-1* heterozygotes. In the presence of a wild-type allele of *Flk-1*, the *SCL* replacement allele greatly boosted BL-CFC numbers. Since it had recently been shown that single Flk-1⁺ cells can have both endothelial and smooth-muscle differentiation potential [82], the effect of SCL on smooth-muscle differentiation was also assayed. In blast colonies from the *Flk-1/SCL* knockin cells, SCL expression was found to bias differentiation towards endothelial at the expense of smooth-muscle development. Together these results suggest actions of Flk-1 and SCL at multiple levels, both at the time of specification of the hemangioblast, and in its subsequent differentiation. Sustained high levels of SCL probably promote hematopoietic differentiation of Flk-1⁺ mesoderm at the expense of a default endothelial program, which itself may require sustained low levels to prevent further differentiation towards smooth muscle. Surprisingly, although HSCs continue to express SCL [83,84], its deletion from the bone marrow via a conditional knockout has no effect on HSC maintenance [85,86]. This indicates that the hematopoietic program initiated by SCL is self-sustaining.

Another interesting genetic regulator, Runx1, also known as Cbfa2 or AML1, is strongly expressed at all hemogenic

sites of the day-8.5 embryo, but its expression is maintained strongly only in intra-embryonic sites [87]. Mutation of *Runx1* blocks definitive but not primitive hematopoiesis, leading to embryonic death by day 12.5 [88,89]. The colony assays described above have been used to study the role of Runx1 in hematopoietic specification. EBs lacking Runx1 have normal numbers of EryP-CFC, but they have much reduced numbers of BL-CFC, and on hematopoietic replating most of these BL-CFC have only primitive erythroid potential [90]. This result prompted the suggestion that there were two types of BL-CFC: one restricted to primitive erythropoiesis independent of Runx1, and the other designated for definitive hematopoiesis, requiring Runx1. Although Runx1 is necessary for definitive hematopoiesis, its expression during primitive erythropoiesis would appear to rule out a role in the specification of definitive vs primitive cell fate. In addition, although it is expressed at intraembryonic hemogenic sites, its expression precedes by many days the acquisition of adult-repopulating potential and so cannot autonomously specify this property either.

Colony assays can also help determine the growth factor requirements of the cell types along the pathway from pluripotent to hematopoietic. In serum-free chemically defined medium, activin A and BMP-4 are able to induce dorsal or ventral mesoderm formation in EBs, respectively [91]. Hematopoietic specification of ventral mesoderm is sensitive to the concentration of BMP-4, with embryonic globin expression occurring within a narrow range. BMP-4 synergizes with VEGF in the generation of CFCs from EBs [92], pointing to a possible role for it at the level of hemangioblast specification. bFGF has been implicated in hemangioblast specification, as it increases BL-CFC output in a concentration-dependent manner, and is moderately enhanced by low levels of activin A [73]. Thrombopoietin, recently shown to play an important role in maintenance and proliferation of the HSC [93–95], and in yolk sac hematopoiesis [96], synergizes with and can actually replace VEGF in the BL-CFC assay, indicative of an important role in hemangioblast development [97]. Wnts have also been studied during EB differentiation, and Wnt3 and Wnt5a, whose expression correlates with the onset of hematopoiesis, are both capable of increasing the output of mixed CFCs when added to the differentiation medium, although whether their effects are mediated above, below, or at the level of the hemangioblast has not been determined [98].

The first system used to differentiate human ES cells into CFCs, which utilized an S17 stromal cell feeder layer [38], has not yet been assayed for growth factor effects. The initial report of growth factor effects on human ES cell differentiation made use of a combined method of EB formation followed by disaggregation at day 5 and growth on fibronectin-coated dishes. After 10 days of growth in the absence of growth factors, globin gene expression was observed by reverse transcriptase polymerase chain reaction (RT-PCR) [99]. Because growth factors were assayed only

on these replated cells, only inhibitors of hematopoiesis were identified in this system: TGF- β blocked globin expression, and activin A moderately inhibited it. CFCs have just recently been documented from human EBs, and their numbers shown to be enhanced by the addition of a cocktail of cytokines (SCF, Flt-3 ligand, IL-3, IL-6, and G-CSF) to the EB culture medium [100]. When BMP-4 was added to the cocktail, both CFC number and colony replating potential were increased, suggesting that BMP-4 acts to enhance the self-renewal of the earliest hematopoietic progenitors. A hemangioblast colony assay has not yet been documented for differentiating human ES cells.

Perils and pitfalls

Analysis of differentiation often makes use of individual markers to identify cell types. In particular, hematopoiesis in the adult is well understood in part because of the availability of a panel of antibodies to cell surface markers specific for distinct lineages and stages of differentiation. When this method of analysis is projected onto the study of differentiating ES cells, it is necessary to keep in mind that specificity of markers defined in the adult does not necessarily imply similar specificity in the embryo. A case in point is the common use of CD45 as a pan-hematopoietic marker. Two recent studies have demonstrated that most hematopoietic CFCs in the day-6 EB are actually CD45⁻, while a marker indicative of megakaryocyte differentiation in the adult, CD41, is pan-hematopoietic at this stage [101,102]. A significant proportion of day-9.5 yolk sac CFCs were also found to be CD41⁺CD45⁻. As development proceeds, CD41 is less frequently expressed by hematopoietic progenitors, and eventually becomes restricted to the megakaryocytic lineage. As CD45 is commonly used to distinguish hematopoietic from nonhematopoietic tissue in developmental studies, all conclusions based on such analysis need to be revisited. This demonstrates the danger of using markers that have not been carefully validated in studies of embryonic tissue.

A related issue is the apparently promiscuous nature of expression of many antigens by embryonic tissue. For example, the BL-CFC is marked by Flk-1, but so are 40% of cells from a day-3.5 EB. PECAM is commonly used as a marker of endothelial differentiation, but it is expressed by 25% of cells in a day-6 EB. VE-cadherin, commonly used for the same purpose, has recently been shown to be expressed by the neonate-repopulating cells of day-9.5 yolk sac and AGM [65]. Although this may be indicative of a hemogenic endothelium at these stages, VE-cadherin also marked progenitors from embryos as early as day 7.5 that were capable of undergoing hematopoiesis on OP9 stroma [65]. The concept of cell type as a strictly uniform entity is probably less appropriate to early stages of embryonic differentiation than to later fetal stages or to the mature organism. When modeling development of pluripotent cells, it is worthwhile to

consider multiple fate-invoking events occurring simultaneously with resulting cascades of gene expression so tuned as to eventually collect into one stable state or another.

Enabling adult repopulation

Because of their inability to engraft adult recipients, it is difficult to say whether the developmental potential of unmanipulated ES-derived blood progenitors is intrinsically limited or simply constrained by their inability to survive in an inappropriate environment. One route to studying the intrinsic potential of such progenitors is to enforce their engraftment with a transforming factor that blocks HSC apoptosis, or drives HSC proliferation, but leaves the lymphoid-myeloid differentiation ability of the HSC unperurbed. The chronic myeloid leukemia-causative oncogene, *Bcr/Abl*, drives HSC proliferation and blocks apoptosis [103,104] and, as shown by the presence of the Philadelphia chromosome in both lymphoid and myeloid cells of patients with CML [105], is permissive for differentiation. When cells from a day-5 EB were transduced with *Bcr/Abl* and plated on OP9 stromal cells, they gave rise to an exponentially growing blast cell population [106]. These cells could be expanded clonally in the absence of stroma, and could differentiate *in vitro* into primitive erythrocytes, suggesting that *Bcr/Abl* had targeted a cell akin to the primitive yolk sac hematopoietic stem cell. The same clones were also able to engraft sub-lethally irradiated adult recipients with a leukemia that contained erythroid, myeloid, and lymphoid components. Furthermore, Ter119-sorted donor-derived erythroblasts from these leukemias expressed adult rather than embryonic globin. This demonstrates that definitive hematopoietic differentiation potential can remain latent in cells exhibiting primitive differentiation potential, and can be uncovered by exposure to the adult environment, provided that the cell can survive in that environment. When ES cells were differentiated immediately on OP9 rather than first being taken through 5 days of EB differentiation, *Bcr/Abl* expression stimulated a different population consisting of myeloid and mixed-lineage progenitors at the expense of erythroid elements [107]. This apparent inconsistency may be due to differences between the respective expression systems used in these reports, but it is more likely that these two methods of differentiation produce somewhat different target cells, which respond differently to *Bcr/Abl* expression.

Hematopoietic differentiation on OP9 has some similarities to EB differentiation, for example the production of an early wave of EryP-CFC followed by a later wave of EryD-CFC [108], but it also has distinctions, such as the facile generation of lymphoid progenitors [109]. The poverty of lymphoid progenitor formation in early EBs, combined with the fact that EBs generate primitive endoderm (the hematopoietic inducing agent for yolk sac mesoderm), suggest that EB differentiation more closely reflects yolk sac hematopoietic induction than does direct differentiation on OP9. The

approach of generating hematopoietic progenitors by short-term EB differentiation followed by culture on OP9 stromal cells is thus similar to exposing yolk sac–derived hematopoietic progenitors to the bone marrow microenvironment. By eliminating the growth factors for the more committed CFCs, and providing growth factors appropriate to the definitive hematopoietic stem cell (stem cell factor [SCF], thrombopoietin [TPO], Flt-3 ligand, and VEGF), the OP9 stromal cultures can be made selective for this therapeutically valuable cell type [54,110]. Under these conditions, cells from wild-type EBs give transient cobblestone area colonies, which do not replat when transferred to secondary OP9 stromal cultures. This is presumably reflective of the inability of the yolk sac–derived HSC to thrive in conditions appropriate to its bone marrow cognate. By inducible expression of various candidate genes in this system, it is possible to screen for factors that might mediate a primitive to definitive fate conversion in the yolk sac HSC.

One candidate to emerge from this approach is the homeobox gene, *HoxB4* [54]. *Hox* genes are expressed in dynamic patterns during adult blood maturation, typically in temporal windows spanning various stages of commitment, with a small subset restricted to the HSC [111]. A comparison of this set with the yolk sac *Hox* gene expression complement [112] yields *HoxB3*, *B4*, *A4*, and *A5* as genes that might be expressed selectively in the definitive vs primitive HSC. *HoxB4* expression during EB differentiation enhances CFC formation [113] and its expression in bone marrow endows the HSC with enhanced self-renewal capacity [114,115]. In the presence of ectopic *HoxB4* expression, day-6 EB cells generate a characteristic cobblestone colony on OP9 whose cells can be expanded exponentially in vitro. After a period of expansion, *HoxB4*-induced cells acquire the ability to engraft in conditioned adult mice and contribute to long-term lymphoid and myeloid differentiation [54]. Continued ectopic *HoxB4* expression is not necessary for graft maintenance once the cells are in the recipient mice, indicating that expression of *HoxB4* in vitro fundamentally alters the potential of these cells. In the first published demonstration of therapeutic cloning, ES cells derived from *Rag2*^{-/-} mice by somatic cell nuclear transfer and subsequent gene correction were differentiated with *HoxB4* expression and shown to generate lymphocytes in *Rag2*^{-/-} recipients [116]. In both cases, the lymphoid contribution was much lower than the myeloid contribution, possibly the result of skewing of differentiation by *HoxB4* [117], but possibly also reflective of the original nature of the cell targeted by *HoxB4*. An incomplete primitive to definitive respecification may also explain why engraftment in wild-type mice is fractional despite the injection of several million OP9-cocultured cells. By using mice lacking functional B, T, or NK cells due to mutation of the cytokine receptor common γ chain [118], engraftment of these cells can be significantly improved [116]. This may implicate NK cells in the rejection of the majority of injected cells since, being derived from ES cells,

they express sub-optimal levels of MHC. Alternatively, the enhanced cytokine milieu of this mutant recipient [119] may improve the viability of the cells during engraftment.

It is striking that transient expression of a single regulatory gene can endow primitive HSCs with significant aspects of definitive hematopoietic potential. This result supports the idea that the primitive HSC is poised to mature into the definitive HSC in response to the appropriate signal. Whether or not *HoxB4* is a physiological regulator of this process remains to be proven—the other group 4 paralogues are similar enough in sequence that they might have the same effect. More interesting than the downstream transcriptional mediators would be the nature of the signal that induces them. Because neither AGM- nor yolk sac–derived tissue has repopulation potential before day 10.5, and because both acquire this potential more or less synchronously, the signal may involve a diffusible, circulating factor. Given the observation that these changes coincide with the capacity of the fetal liver to support hematopoiesis, and indeed with the rapid accumulation of HSC activity within the fetal liver between days 11 and 13, some component of the developing liver may be the source of such a factor. A summary of the functions of various cell-intrinsic and -extrinsic regulators of hematopoietic development is given in Figure 3.

Prospects for the future

The derivation of human ES cells and the prospect of using them for therapy has brought new importance to understanding the origins of the hematopoietic lineage and the events driving the transition from primitive to definitive hematopoiesis. The sooner these processes can be modeled in vitro from human ES cells, the closer we will be to a viable therapy. Recapitulating the work that has been done in the murine system using human ES cells will not necessarily be straightforward. Spontaneous blood development in human EBs is not as obvious as it is in mouse EBs [120]; however, the fact that human ES cells can generate CFCs is encouraging [38,100]. Surface antigen expression and trophectodermal differentiation capacity suggest that the cell type represented by the currently available human ES cell lines is a developmental antecedent to the cell type represented by murine ES cells [121], and therefore not necessarily subject to identical rules of differentiation. In addition to the differences between human and murine ES cells, everything that we have learned from the murine system suggests that the engraftment potential of hematopoietic progenitors spontaneously derived from human ES cells will be poor to negligible without developmental maturation. It will be interesting to learn how human ES-derived progenitors respond to *HoxB4* expression and, if and when extrinsic factors mediating primitive to definitive hematopoietic fate switching are identified, how they respond to such factors. Regardless of how easily human ES cells recapitulate the performance of their murine counterparts, it is clear that our

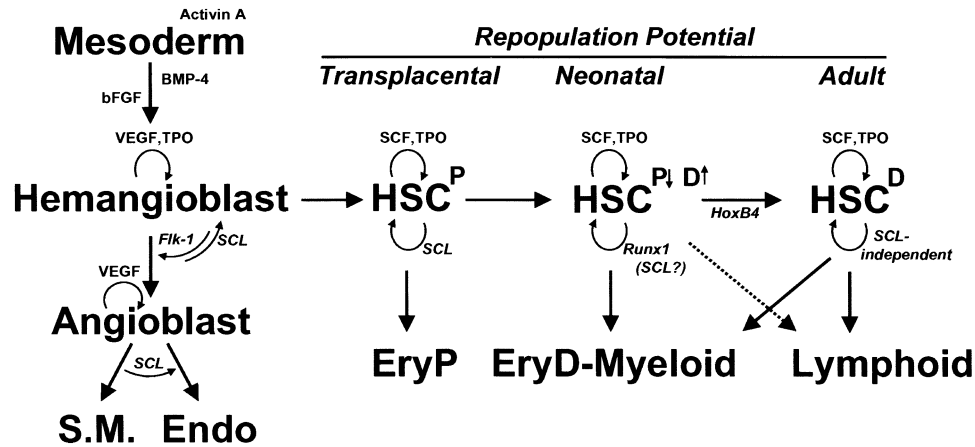


Figure 3. Origin and developmental maturation of the HSC. Cytokines promoting maintenance of each cell type are shown above; transcriptional programs required for maintenance are shown below. Hematopoietic, endothelial, and vascular smooth-muscle cells all derive from a common mesodermal precursor, the hemangioblast. Mesodermal specification towards the hemangioblast may involve activin A, bFGF, and/or BMP-4. The hemangioblast expresses both Flk-1 and SCL with SCL promoting hematopoietic commitment and Flk-1 signaling promoting endothelial. Low residual levels of SCL inhibit the differentiation to vascular smooth muscle in the endothelial arm. The hematopoietic arm matures in stages, each defined by its immediate differentiation potential, denoted below, and its transplantation competence, denoted above. The first stage is the primitive HSC, which transiently gives rise to the primitive erythroid lineage. As maturation progresses, the HSC loses the capacity to generate primitive erythrocytes and acquires the ability to generate definitive erythrocytes and myeloid cells. Although no longer primitive, neither is it fully definitive, as it is unable to repopulate adult recipients and its lymphoid potential is limited. This cell type represents both yolk sac–derived and AGM-derived HSC at about day 10 of embryogenesis. The controversial lineage relationship between these two cells is discussed in the text; however, those AGM-derived HSCs that are autonomously generated, must skip the primitive HSC stage. An as-yet-unknown signal induces the final maturation step. This final maturation step can be partially recapitulated by HoxB4 expression.

ability to distill the therapeutic hematopoietic potential of ES cells will continue to advance hand-in-hand with our understanding of hematopoietic ontogeny.

Acknowledgments

We thank Rita C.R. Perlingeiro and M. William Lensch for helpful comments on the manuscript. GQD is the Birnbaum Scholar of the Leukemia and Lymphoma Society of America. Research support was received from the Alberta Heritage Foundation for Medical Research, the Canadian Institutes of Health Research, the National Institutes of Health (grants CA76418, CA86991, DK59279, and HL71265), and the National Science Foundation–MIT Biotechnology Process Engineering Center.

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