The mysterious genesis of hematopoietic stem cells during early vertebrate development has intrigued investigators for several centuries. The molecular steps that lead to embryonic hematopoiesis remain to be determined, although recent studies have begun to define early events and migration patterns that regulate the hematopoietic program. This review will provide a historical background of the developmental biology of vertebrate hematopoiesis and highlight the systems and methods that are being used to study the regulation of the hematopoietic stem cell during embryogenesis.

The ontogeny of primitive and definitive hematopoietic cells

During vertebrate ontogeny, hematopoietic sites change as new populations of stem cells emerge. In mammalian development, stem cells sequentially occupy the embryonic yolk sac, fetal liver, spleen, and adult bone marrow (BM). The factors that regulate homing to hematopoietic sites have not been identified, yet lower vertebrates provide some information about the process. The BM is first used in evolution as a site of adult hematopoiesis by certain amphibians (Rana species but not Xenopus). Seasonal changes can cause a shift in sites of hematopoiesis in some amphibians (between the spleen and BM), possibly because of alterations in circulating steroid levels. Although the yolk sac, fetal liver, spleen, and BM are common sites of hematopoiesis in many vertebrates, their use is not required, and some organisms have developed unique characteristics of these hematopoietic sites. The chicken does not use the fetal liver as a major erythropoietic organ but instead maintains blood formation on the yolk sac and in the mesenteric regions until BM hematopoiesis is established. In some organisms, hematopoiesis occurs in unusual places such as the ovary, testes, adrenal glands, and endocardium. Therefore, hematopoietic stem cells possess the ability to migrate within the organism to a region that will support their existence and allow subsequent differentiation.

In early studies of hematopoiesis, a model was suggested in which fetal and adult hematopoietic stem cells are derived from the original embryonic blood stem cells that occupy the early yolk sac (Fig 1, top). In this model, a yolk-sac stem cell migrates or "metastasizes" to a fetal hematopoietic site, such as the liver, where it expresses a fetal program on differentiation. Therefore, the activation of the fetal or adult program relies predominantly on transcriptional regulation, which could be triggered by environmental cues.

Although this model had certain appeal, other studies showed that embryonic blood cells represent a distinct population of cells from the fetal hematopoietic cells (Table 1). For instance, embryonic erythrocytes morphologically are larger in size, are nucleated, and have a more generous cytoplasm than fetal/adult blood cells. Embryonic hematopoietic cells are mostly restricted to the erythroid lineage in vivo, although this may partly be due to characteristics of the hematopoietic microenvironment. Embryonic cells are constantly cycling during development and do not pause for very long periods of time to differentiate, whereas fetal and adult hematopoietic stem cells remain in G0 for extensive periods of time and are only activated occasionally, when needed. Finally, embryonic, fetal, and adult hematopoietic cells each synthesize distinct globins. These differences suggest a model (Fig 1, bottom) in which the embryonic ("primitive") and fetal/adult ("definitive") hematopoietic stem cells are distinctly derived early in development. Primitive cells undergo programmed cell death during fetal life, and only definitive progenitors give rise to fetal and adult hematopoiesis (Fig 1, bottom). A corollary to the primitive-definitive model suggests that globin switching is largely governed by changes in cell populations rather than transcriptional events.

Induction and migration of hematopoietic stem cells and progenitors

Hematopoietic stem cells are derived from ventral mesoderm

To comprehend the early events that lead to embryonic hematopoiesis, it is necessary to understand pattern formation of the early vertebrate embryo. Classical studies in developmental biology have used amphibians to examine embryogenesis (see Figs 2, 3, and 6), and the general principles of embryonic development are maintained in higher organisms. The unfertilized Xenopus egg contains an animal pole (top hemisphere by convention) and a vegetal (bottom hemisphere) pole. After fertilization, the dorsal axis of the embryo will arise opposite the sperm entry point, and by the 32-cell stage, a single ventral cell (the C4 blastomere, Fig 2) is fated to become blood. The early events of embryogenesis are driven solely by maternal determinants deposited in the egg during oogenesis. The mid-blastula transition defines a developmental period, approximately 7 hours after fertilization, when maternal mRNAs degrade and zygotic transcription initiates. An early vertebrate embryo at the blastula stage consists of two germ layers, (1) ectoderm, which is located in the animal pole and is fated to become skin and neural tissues, and (2) endoderm, which is located in the vegetal pole and later becomes the gut. During gastrulation, morphogenetic movements (invagination just below the equator of the embryo at the blastopore lip) brings ectoderm into apposition with endoderm. By the elaboration of
DEVELOPMENTAL BIOLOGY OF HEMATOPOIESIS

Two developmental models for hematopoiesis. In the top model, embryonic hematopoietic stem cells (P) migrate from the yolk sac to the fetal liver, where they differentiate into fetal hematopoietic stem cells (D). Later, the second switch occurs when the fetal liver stem cells migrate to the BM. In the bottom model, embryonic hematopoietic stem cells (P) exit the yolk sac and undergo programmed cell death. Another distinct population of cells (D) migrates from the yolk sac to the intra-embryonic dorsal mesentery or fetal liver and becomes the definitive pool. Some definitive cells from the fetal source can also migrate to the embryonic site. P, primitive cells; D, definitive cells.

Table 1. Comparison of Embryonic, Fetal, and Adult Erythropoiesis

<table>
<thead>
<tr>
<th>Lineages</th>
<th>Embryonic</th>
<th>Fetal/Prehatching</th>
<th>Adult</th>
</tr>
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<tbody>
<tr>
<td>Stem cell</td>
<td>Erythroid</td>
<td>Stem cell</td>
<td>Erythroid</td>
</tr>
<tr>
<td>H</td>
<td>Yolk sac</td>
<td>Yolk sac</td>
<td>Liver</td>
</tr>
<tr>
<td>M</td>
<td>Yolk sac</td>
<td>Yolk sac</td>
<td>Liver</td>
</tr>
<tr>
<td>C</td>
<td>Yolk sac</td>
<td>Yolk sac, diffuse</td>
<td>Liver, (kidney)</td>
</tr>
<tr>
<td>X</td>
<td>Ventral island</td>
<td>Liver, (kidney)</td>
<td>Spleen, liver, spleen</td>
</tr>
<tr>
<td>F</td>
<td>IM</td>
<td>Pronephros</td>
<td>Kidney, liver, spleen</td>
</tr>
</tbody>
</table>

Nucleated RBC

| H | Yolk sac | Yolk sac | Liver | BM |
| M | Yolk sac | Yolk sac | Liver | BM, spleen |
| C | Yolk sac | Yolk sac, diffuse | Liver, (kidney) | BM, spleen, (BM) |
| X | Ventral island | Liver, (kidney) | Spleen, liver, spleen |
| F | IM | Pronephros | Kidney, liver, spleen |

Only major globins are listed; eg, murine β4 globin is a low-abundance variant of β4, globin. The values shown in parentheses indicate site(s) used by some amphibian species.

Abbreviations: H, human; M, mouse; C, chicken; X, Xenopus; F, fish (teleosts); RBC, red blood cells

Hematopoietic stem cells that ultimately differentiate into erythroblasts, and an endothelial layer that surrounds the blood island as it is developing (Fig 4, bottom).

Mammals. In the mouse, on day 7½, blood island formation begins in a central area of the egg cylinder. At day 7½, there are regions of thickening of the inner mesodermal layer in contact with the endoderm, which is thought to support the growth of the hematopoietic tissue. Endothelial cells subsequently differentiate and encompass the hematopoietic progenitors, which then bud off of the endodermal base, forming a blood island. By day 9, there is a capillary network, and mature erythroid cells enter circulation, but yolk sac blood formation proceeds through day 12. In hu-

Yolk Sac Hematopoiesis

In most vertebrate organisms, hematopoietic stem cells and vascular progenitors migrate to an extra-embryonic position on the yolk sac (which is considered ventral by embryologists) to form the blood islands. Later, these cells enter the embryo to form the primitive and definitive hematopoietic lineages and a subset of the vasculature, respectively (Fig 4).5,11,15 A blood island consists of at least three layers: an endodermal layer that supports growth, a central core of soluble peptide growth factors or by cell-cell interactions, the endoderm induces ectoderm to form a third germ layer known as mesoderm. This layer is initially located between the two other germ layers (ectoderm and endoderm) in a middle layer called the marginal zone. Mesoderm is the undifferentiated, self-renewing cells of the embryo that will provide stem cells for the following tissues (from ventral to dorsal across the marginal zone): blood, mesenchyme, kidney, muscle, and notochord. Therefore, blood development involves the induction, proliferation, and differentiation of ventral mesoderm.

Fig 2. A 32-cell fate map of the Xenopus embryo. This was constructed by injection of various dyes into a single cell (also called blastomere) of each tier of the 32-cell embryo and following the dye as development proceeds. The C4 blastomere is the predominant cell fated to form blood (Reprinted with permission.)"
mans, the process is similar to that for the mouse; hematopoiesis occurs initially at day 15 and continues for 6 weeks of gestation (Fig 4). Mammalian embryonic erythroid cells are nucleated, similar to the erythroid cells of lower vertebrates including fish, frogs, reptiles, and chickens. Although most progenitor populations are present in the yolk sac, the terminal differentiation occurs primarily to the erythroid lineage.21,22 Occasionally, monocytes and megakaryocytes have been identified in blood islands.

**Birds.** The early embryology of blood formation has been well-defined in the chicken (Fig 5A, B, and C).22 The boundary of the embryo is not distinct until after primitive stage development, but the area opaca is considered extra-embryonic even from early stages (Fig 5A). Cells originating from the epiblast (the upper layer) invaginate through the primitive streak and enter the area pellucida between the epiblast and hypoblast (the lower layer) by 15 to 16 hours. The cells are later separated into somatic and splanchnic layers. Extra-embryonic splanchnoderm consists of two different layers, an endodermal layer and a mesodermal layer that will ultimately give rise to blood islands in the yolk sac. The vascular area invades the vitelline area, the two layers become indistinct, and, eventually, the entire yolk sac is vascularized. The initial blood islands are seen in the posterior area opaca (Fig 5B) at the head-process or early somite stages, at which time the posterior area pellucida becomes vascularized. Hemoglobin can be detected as early as the 2- to 4 somite stage.22 As ventral migration occurs, the lateral plate mesoderm converges between the blood cells and skin and yields the endothelium necessary for circulation.

**Amphibians.** Amphibians form blood in the ventral region of the embryo (Fig 6A). Brauns32 described the prospective blood island region at early neurula stage as a monolayered or bilayered sheets of cells extending along the entire ventral region of the embryo. At first, these cells migrate slowly in a ventral direction, then quickly migrate to form the characteristic hematopoietic cords of blood island in a "V" shape (Fig 6B). As ventral migration occurs, the lateral plate mesoderm converges between the blood cells and skin and yields the endothelium necessary for circulation. Excision of this region during neurula stages leads to tadpoles with no circulating blood within a normal circulatory system.22,23 Bloodless tadpoles can live for over 1 month, indicating that blood is not critical to early embryonic development in amphibians.

**Fish.** Bony fish (teleosts) form embryonic erythroid cells in a distinct dorsal-lateral compartment of the embryo known as the intercellular mass (IM) of Oelachfer33 (described in 1872; see Fig 7), and are, therefore, an exception to the general vertebrate rule of embryonic hematopoiesis on the yolk sac.22-23 The IM is derived from lateral mesoderm at the posterior border of the embryo and is similar in position to the initial yolk sac-derived hematopoietic mesoderm in higher vertebrates.32 Some Teleosts such as the angelfish, killifish, and cartilaginous fish form embryonic blood both on the yolk sac and in the IM.23

In Xenopus, hemoglobinization occurs in an anterior-to-posterior direction34 starting at 36 hours after fertilization. The island is anteriorly bounded by hepatic endoderm, which is thought to secrete erythropoietin-like activity that accounts for the anterior-to-posterior wave of hemoglobinization. The heart starts beating at stage 33/34 (45 hours), the anterior cells of the blood island enter the heart from the primitive vasculature, and circulation is fully established at stage 35/36 (50 hours). The immune system begins to develop between stages 42-45 (80 hours), when the thymus becomes histologically visible.

**Dorsal Hematopoiesis.** In addition to ventral embryonic (or yolk sac) hematopoiesis, most vertebrates have an additional intra-embryonic stem cell population in the dorsal mesentery (also called the AGM region for aorta, gonad, mesonephros region) that colonizes later fetal (larval) sites of blood formation. In higher vertebrates, this dorsal population predominantly gives rise to
Fig 5. Avian embryonic blood formation. (A) Early mesoderm induction until 15 to 16 hours. The invagination of mesoderm through the primitive streak is depicted in a cross-section. (B) Chicken embryonic hematopoiesis at the 7-somite stage. (Adapted and reprinted with permission.) Note that the initial hematopoietic progenitors arise in the area opaca.
definitive hematopoiesis, but in lower vertebrates both primitive and definitive cells arise from these cells.

Studies using an in vitro embryo culture assay show that the yolk sac is required for both primitive and definitive hematopoiesis in mammals. Culture of day-7 (early 1-4 somite) mouse embryos in tissue-culture plates showed normal somite development, yolk sac hematopoiesis, and a beating heart. Culture of day-7 yolk sac alone yielded abundant hematopoietic colonies, but culture of day-7 embryos from which the yolk sac has been removed develops without blood in a normal circulatory system. In some of these embryos, there was an absence of fetal liver hematopoietic progenitors, suggesting that the genesis of the definitive hematopoietic program requires the yolk sac structure. Therefore, the primitive and definitive lineages are likely to be derived from common progenitor cells colocalized in the yolk sac (IM or ventral blood island) region very early during embryogenesis.

Fig 5. (Cont'd) (C) Expansion of yolk sac hematopoiesis from day 1 (top embryo), day 2 (second), day 3 (third), and day 4 (bottom embryo). (Adapted and reprinted with permission.)
Fig 6. Xenopus embryonic blood formation. (A) Schematic view of hematopoietic induction and development (Adapted and reprinted with permission).

The Xenopus egg contains an animal and a vegetal pole that are distinguishable by pigment. The C4 blastomere of the embryo that will become blood is determined as early as the 32-cell embryo (shaded). Presumably, the vegetal blastomere under this shaded region is responsible for the induction of ventral type mesoderm. Mesoderm is derived from the ectodermal animal pole. Two predominant signals have been postulated to affect the process of mesoderm induction, the ventral signal (V) and the dorsal signal (D). During gastrula stages, the Spemann organizer (arrow) functions to pattern mesoderm across the marginal zone (the central region of the embryo). The commitment to form blood occurs in the ventral region of the marginal zone. Later in neurula and tailbud stages, blood is the most ventral mesoderm, and is located adjacent to the epidermal layer. (B) Xenopus embryo at stage 28 (about 34 hours after fertilization). This embryo is stained for embryonic $\alpha$-globin using whole embryo immunohistochemistry analysis.
The migration of hematopoietic progenitors during and after neurula has been extensively studied in amphibians. By transplanting a cytogenerically distinct ventral blood island tissue into wild-type hosts, a small ventral portion of the Xenopus neurula embryo has been shown to contain primitive and definitive erythroid and T-cell/myeloid progenitors (Fig 8A).6,7,34-42 This region in Rana pipiens contributes to primitive erythropoiesis but does not contribute to T-cells or myeloid cell populations.43-46 In Rana catesbeiana, the definitive progenitors of the dorsal mesentery colonize the larval liver, whereas the primitive progenitors of this region colonize the larval pronephros.47 Thus, the distribution of progenitor cells in the ventral or dorsal compartment of the embryo is not conserved during vertebrate evolution, even in closely related species. Cell transplantation studies have also shown that T-cell progenitors are derived from the posterior region of the embryo during neurula stages (Fig 8B), and these cells migrate anteriorly across tissue planes to eventually colonize the dorsal aorta region, the postcardinal veins, and the thymus. The thymus may also be colonized by circulating cells. The migratory pattern of hematopoietic cells throughout amphibian development is schematically drawn in Fig 8C.

The embryonic region to which the stem cells are transplanted influences the frequency of contribution to definitive cell lineages (Fig 8D).56 Lymphoid contribution is significantly increased when cells are transplanted to a peripheral location in the embryo, whereas erythroid contribution is increased when cells are placed in a central region. Hence, environment apparently exerts a directive effect on stem cells of the embryo to form particular hematopoietic tissues.

The embryonic location of definitive hematopoietic progenitors in birds has been studied with interspecies grafts between chickens and quails, so-called “yolk sac chimeras.”43 In these experiments, age-matched chicken and quail eggs are incubated for 30 to 36 hours (between 8-12 somites), and the quail embryonic body is grafted into the analogous position of a chicken blastoderm. The quail embryo develops on the yolk sac, and, at varying developmental stages, quail cells are recognized by morphological differences or by markers such as immunofluorescence with the quail-specific monoclonal antibodies, QH148 or MB1.49 QH1 and MB1 recognize glycoproteins on quail hematopoietic, vascular, and germ cell lineages, but not on chicken cells.

Between d3 and d5, chickens and quails have hematopoietic foci on the ventral wall of the aorta.4,5,50 These foci may be induced by endoderm that physically associates with the ventral surface of the two dorsal aortae before they fuse.31 By day 6-8, the definitive hematopoietic progenitors in chicken and quail embryos are distributed differently in the mesenchyme (Fig 9A). In the chicken, definitive hematopoietic foci are found throughout the mesentery and around visceral organs, including the dorsal region that will become the thoracic duct. In the quail embryo, hematopoietic tissue is found associated with the anterior cardinal vein system, at the angle of the duct of Cuvier. These dorsal hematopoietic cells are only separated from the vascular lumen by endothelium and can infiltrate the wall of large venous vessels. Using the QH1 marker, cells from the dorsal regions can be followed as they colonize the yolk sac or thymus by direct migration or as they differentiate in situ.

The dorsal hematopoietic program of avians is regulated by local environment. At day 3-4, mesodermal cells surrounding the quail dorsal aorta can be reciprocally transplanted to the analogous position in the chick embryo.51 The donor quail cells contribute to blood-forming foci and, occasionally, to endothelial structures based on QH1 staining. When day 3-4 mesodermal cells are cultured with day 6.5 chick thymic rudiment, the cells stain with QH1 and also react with the CT1 antibody (a T-cell marker). This suggests that the mesoderm adopts a T-cell program in the environment of the thymus.

Recently, similar dorsal hematopoietic cell populations have been shown for mammals (Fig 9).52-54 Spleen colony-forming unit activity was shown in the region of the aorta, gonad, and mesonephros from day-8 to day-11 embryos. By grafting intra-embryonic splanchnopleura from 10- to 18-somite embryos in SCID mice, IgM-secreting plasma cells and the B1a cell subset were reconstituted. Thus, the dorsal compartment in mice is ultimately capable of hematopoietic development as documented for early spleen colony-forming unit and B-cell development.
A General Hypothesis for Migration Pathways of Hematopoietic Cells During Vertebrate Embryogenesis

During early gastrulation, induced ventral mesoderm migrates to a lateral position on the yolk sac. As gastrulation proceeds, these cells either migrate further onto the yolk sac or return to an intra-embryonic location in the dorsal mesentery. Most cells on the early yolk sac are primitive progenitors that will form blood islands and eventually enter the circulation. As the fetus develops, these primitive cells undergo programmed cell death. Fetal-adult blood cells are derived either from definitive progenitors that colonized the yolk sac at the same time as the primitive progenitors or from the dorsal mesenteric hematopoietic cells. The dorsal mesenteric cells either migrate to the yolk sac or thymus or can enter the circulation and colonize hematopoietic sites such as the fetal liver. Thus, the prominent waves (from early to late) of hematopoiesis in the embryo include (1) primitive cells on the yolk sac, (2) definitive cells on the yolk sac (which are likely derived from ventral mesoderm that migrated with the primitive cells), and (3) early intra-aortic and later para-aortic hematopoietic cells. Some of the dorsal hematopoietic cells migrate to the yolk sac and form a later wave of hematopoiesis on the yolk sac. These migratory patterns of hematopoietic cells in the vertebrate embryo are generally conserved throughout evolution.

Hematopoietic and Endothelial Development

The vascular and hematopoietic compartments are coordinate regulated so that blood cells enter the circulation as soon as terminal differentiation has occurred. A common progenitor called either a “hemangioblast,” “hemangioblast,” or “angioblast” has been postulated to exist during embryogenesis and has the potential to form either vascular endothelial cells or hematopoietic cells in the blood island region. Morphological data derived from experiments with cultured chicken blastoderm support the hypothesis that these bipotential cells can form either tissue. In mice and humans, support for this concept is less apparent. Support for the existence of a common blood/vascular progenitor includes the finding of antigens (such as QH1) or molecular markers (such as GATA-2 and several tyrosine kinases) that are common to early hematopoietic and endocardial progenitors, potentially representing a defect in a common blood-vascular progenitor. Despite the above data supporting the existence of a hemangioblast, the vasculature and heart develop normally in bloodless amphibian embryos from which the presumptive ventral blood island region has been explanted during the neurula stage. It is possible that, by early neurula, fate has been determined and the vascular lineage becomes distinct from the hematopoietic lineage; most ventral mesoderm becomes hematopoietic, whereas the lateral mesodermal cells become the vascular progenitors.

STUDIES ON LOWER VERTEBRATES

We have used the fish and frog as model systems to study the induction of the hematopoietic stem cell during ontogeny. The embryos from these species are more accessible than higher organisms, and their early development has been well-characterized. The study of their hematopoietic program, which may be more simple, will provide general principles regarding hematopoiesis in vertebrates.

Zebrafish Hematopoiesis

We have characterized the expression of the zebrafish GATA-1 and GATA-2 during early development. These transcription factors are initially induced during gastrulation and delineate cells that will form the hematopoietic IM (Fig 7). The posterior region of the IM contains early hematopoietic progenitors (or “stem” cells) that express GATA-2, but not GATA-1. Thus, the visibility of the zebrafish embryo allows easy examination of early and late hematopoietic populations. Studies of genetic mutants for blood formation in the zebrafish system may provide insight into the induction events that effect hematopoiesis. Using GATA-1 and GATA-2 as markers, we have studied three zebrafish mutants of blood formation. The ventral blood island (VBI) contributes to the development of all hematopoietic lineages. Primitive cells form the blood island and enter the circulation (1). During neurula stages, mesoderm around the pronephric duct (PD) migrates anteriorly (2) to the region of the dorsal aorta (DA; 3) and ducts of Cuvier (DC; 3) and the pronephros (P; 4). These cells either colonize the thymus (5) or enter circulation through the aorta or ducts of Cuvier (6). Definitive cells within the VBI enter circulation also and eventually colonize the thymus (7; T) and the larval liver (LL; 8). Definitive cells also enter the circulation from the larval liver (9). In chickens and mice, the circulation is established before the yolk sac structure is disrupted. Some definitive cells in the dorsal mesentery or in the circulation colonize the yolk sac. The chicken does not form blood in the fetal liver (FL), but has extensive hematopoiesis throughout the mesentery. The axis of the embryo is marked as follows: A, anterior; P, posterior; D, dorsal; V, ventral. (D) Environmental influences of definitive hematopoietic commitment. When the ventral tissue is transplanted to a ventral central location, the donor tissue becomes erythroid, whereas a transplant to a lateral location becomes T cells.
**A**

**Neurula**

<table>
<thead>
<tr>
<th>Species</th>
<th>Neurula Region</th>
<th>Primitive RBC</th>
<th>T-cell</th>
<th>Definitive RBC</th>
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<tr>
<td>Xenopus</td>
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<td>-</td>
<td>+</td>
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<td>Xenopus</td>
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<td>Ventral</td>
<td>+</td>
<td>-</td>
<td>?</td>
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</tbody>
</table>
dendritic staining, and immunolocalization with chicken has diffuse definitive hematopoiesis, particularly around the aortic focus of birds (A) and mammals (B). This region has been shown to be a site of hematopoiesis by morphological criteria, benzidine staining, and immunolocalization with chicken antisera (in birds) and by spleen colony assays and transplantation experiments. The chicken has diffuse definitive hematopoiesis, particularly around the aorta (dark shaded region), whereas the quail has a more restricted distribution of hematopoietic cells surrounding the Ducts of Cuvier (dark shaded region). The hematopoietic progenitor cells in the embryo in situ analysis shows that spadetail formation: bloodless, spadetail, and cloche. The bloodless mutation only affects hematopoiesis, whereas the spadetail mutation affects muscle and blood induction. Whole embryo in situ analysis shows that bloodless (Fig 10) and spadetail mutant embryos each lack GATA-1 and GATA-2 expression, except for wild-type levels of GATA-2 expression in the posterior "stem" cells. Despite their similarities, complementation analysis has shown that the bloodless and spadetail mutations are in distinct genes. Thus, the self-renewal or differentiation of early hematopoietic cells is affected in the mutants.

The cloche mutation lacks the endocardial layer of the heart and the blood cells in the IM region. Interestingly, cloche mutants do not express GATA-2 or GATA-1, even in the posterior stem cells. Thus, the cloche mutation affects vascular cells and hematopoietic cells and, therefore, supports the existence of a bipotential "hemangioblast" during development. Fate mapping experiments in wild-type embryos at the 500-cell stage have shown that single ventral blastomeres give rise to blood and blood vessel progenitors, further suggesting a common origin. Therefore, the defect in cloche effects the genesis of hematopoietic (and endothelial) stem cells.

Two independent laboratories, Driever (Boston, MA) and Nusslein-Volhard (Tubingen, Germany), have produced over 10,000 zebrafish mutations using chemical mutagenesis. In this method, fish were treated with ethyl nitroso urea (ENU), a chemical that causes point mutations in the sperm genome. These sperm were used to fertilize eggs, the progeny were mated individually to wild-type fish or to the parent, and a family was derived. Each family is then individually screened for mutant phenotypes, and over 30 mutants affecting the blood system have been characterized.

The mutants can be used to genetically order a cascade of steps for embryonic blood formation. This is a similar to drosophila or yeast genetic epistasis analysis, which involves paired matings of different mutants, thereby generating fish that are mutant at two (or three) genetic loci. Assessment of the phenotype of these double mutants allows a positioning in a cascade of developmental events. These studies are coupled with the examination of gene expression with specific markers in mutant embryos and with the analysis of the effect of forced gene expression. A zebrafish genome map consisting of over 400 random amplified polymorphic DNA (RAPD) markers distributed throughout the genome has recently been published, and we have started to map the location of the genes affected in the mutant zebrafish. A position-cloning strategy to isolate genes that are affected in the mutants is then possible.

*Fig 9. Schematic diagram of hematopoiesis in the dorsal para-aortic focus of birds (A) and mammals (B). This region has been shown to be a site of hematopoiesis by morphological criteria, benzidine staining, and immunolocalization with QH1 antisera (in birds) and by spleen colony assays and transplantation experiments. The chicken has diffuse definitive hematopoiesis, particularly around the aorta (dark shaded region), whereas the quail has a more restricted distribution of hematopoietic cells surrounding the Ducts of Cuvier (dark shaded region). The hematopoietic progenitor cells in the mouse have been studied functionally (see text) but have not been morphologically or histologically defined. DC, ducts of Cuvier; A, aorta; E, esophagus; B, bronchi. (Adapted and reprinted with permission.)*

formation: bloodless, spadetail, and cloche. The bloodless mutation only affects hematopoiesis, whereas the spadetail mutation affects muscle and blood induction. Whole embryo in situ analysis shows that bloodless (Fig 10) and spadetail mutant embryos each lack GATA-1 and GATA-2 expression, except for wild-type levels of GATA-2 expression in the posterior "stem" cells. Despite their similarities, complementation analysis has shown that the bloodless and spadetail mutations are in distinct genes. Thus, the self-renewal or differentiation of early hematopoietic cells is affected in the mutants.

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**Xenopus Hematopoiesis**

The induction of hematopoietic stem cells during embryogenesis is likely to be regulated by signals that affect mesoderm induction and patterning. These signals may not function in the same manner as hematopoietic cytokines; mesoderm-inducing factors are thought to induce distinct tissues in a gradient rather than threshold mechanism. It is possible that low doses of particular inducing factors would stimulate blood formation, but higher doses may inhibit hematopoiesis. Mesoderm induction has been so well-characterized in Xenopus that this species is ideal to characterize factors that regulate the early events in hematopoiesis.

**Assays in the frog for the induction of hematopoietic stem cells.** Transplantation studies have shown that Xenopus animal-pole ectodermal cells can contribute to almost any tissue of a developing embryo. As such, animal pole cells (also called animal caps) are similar to murine embryonic stem cells. Through the use of animal pole-vegetal pole "recombinants" (see Fig 11), Nieuwkoop and colleagues have shown that the dorsal-ventral organization of mesoderm is determined by the vegetal pole. Culture of animal pole alone in a simple salt solution results in ciliated epidermis (skin), whereas culture of the vegetal pole yields undifferentiated cells and gut (Fig 11). A recombinant of animal and vegetal pole yields mesodermal tissues, including some...
blood cells. During blastula stages, two predominant mesoderm-inducing signals are thought to act on overlying eutorial cells. The ventral vegetal signal induces ventral mesoderm such as blood and mesothelium and the dorsal vegetal signal induces dorsal mesoderm including muscle and notochord. Subsequently, during gastrulation, a dorsal region called the Spemann organizer patterns mesoderm across the marginal zone.88 These data suggest that the endoderm elaborates a signal required for the induction of blood, and that the program is modulated by cell-cell interactions.

Surgical excision and culture of the dorsal marginal zone (DMZ) yields mostly notochord and muscle, whereas ventral marginal zone (VMZ) yields predominantly blood and mesenchyme (Fig 11, bottom).70 VMZs express globin after 40 hours in culture, and, thus, the inducers of blood are available or programmed in this ventral region. A VMZ culture can be viewed as equivalent to hematopoietic progenitor assays with all of the authentic growth factors present.

Interspecies grafts between two different amphibian species (Axolotl and Xenopus) have been used to characterize the ventral and dorsal signals. Culture of Xenopus VMZ with Axolotl DMZ yielded mostly Xenopus muscle and kidney differentiation, showing the respecification of ventral mesoderm by the dorsal signal. If the organizer region of the DMZ is grafted onto the ventral region of an intact embryo (a classic “Spemann Organizer” graft), a mirror-image duplication of the embryo is created.70 Neither the primary nor the secondary embryo is actually complete because they both lack blood islands. Thus, it is possible that the organizer can reprogram ventral mesoderm and, thereby, functionally repress blood formation.

The ventral and dorsal signals have been shown to be soluble factors based on their ability to cross a millipore filter placed between embryonic explants.71-73 Very low levels of globin expression are detected in animal-vegetal recombinants, but not to the level in a VMZ explant.4,74 This suggests that globin production requires signals in the ventral-most endoderm of the marginal zone.76

Studies of mesodermal inducing factors in Xenopus. Activins are members of the transforming growth factor (TGF-β) superfamily and are dimers of inhibin β chains.77 Activin is a very potent inducer of mesoderm in animal cap assays75-78 and can also induce differentiation of erythroleukemia cells.80 Whereas recombinants between animal and vegetal poles yield all mesodermal components, activin A (either from Xenopus or mammalian sources) induces mostly notochord, muscle, and kidney tubules. At low concentrations of 0.1 to 1 ng/mL, activin induces some blood-like cells.77,78 However, the blood cells do not express hemoglobin based on immunofluorescence with an anti-Xenopus globin antisera.

Fibroblast growth factor (FGF) treatment of animal pole explants at low concentrations mimics the effects of the ventral vegetal signal in vivo.86-91 This consists of a concentric arrangement of mesenchyme and mesothelium. Some blood-like cells are present that do not express hemoglobin but have a characteristic morphology.74,75,92 Other growth factors effect the induction of hematopoietic mesoderm and ventral patterning. BMP-4 is a member of the TGF-β superfamily and is capable of ventralizing whole embryos and increasing the expression of globin.93-95 WNT-8, a member of the wnt family, also can ventralize tissues when expressed correctly after the onset of zygotic transcription96-97; however, if expressed earlier, WNT-8 predominantly dorsalizes tissues. More research is needed to determine the exact effect these proteins have on hematopoietic induction, but it is clear that dose and timing of exposure to stem cells will be critical variables to explore. There are also several dorsalizing factors including TGF-β, wnt, and noggin96-100 polypeptides that have been described in Xenopus embryos that may function to suppress hematopoiesis.

Studies of embryonic hematopoiesis in Xenopus. Using whole embryo in situ analysis for GATA-1 and SCL RNA expression, we have shown that the hematopoietic program initiates at least as early as 11 hours after fertilization (during gastrulation).101 GATA-1 RNA was not initially detected in uninduced animal cap cells, but, after 17 hours of culture, GATA-1 was surprisingly expressed.102 The level continued to increase until 25 hours and then decreased. Later in culture, embryonic α-globin was also expressed at a low level in the animal cap cells. The expression of these genes is in accord with the normal temporal pattern of expression. MyoD, a muscle-specific transcription factor, is also expressed in the uninduced animal caps early in the culture and is downregulated so that caps at 48 hours do not express MyoD.103 Thus, the mesodermal programs (such as blood and muscle) are evident in the animal cap cultures, but, in the absence of induction, the programs are not maintained.
Activin or FGF induction of animal pole explants leads to the maintenance of MyoD expression and formation of muscle; however, neither factor rescues the blood program or maintains GATA-1 expression. As discussed above, the ventral vegetal signal(s) present in VMZ explants is capable of inducing and maintaining the blood program as defined by GATA-1 and embryonic α-globin expression (Fig 12). The animal cap itself has also been postulated to affect hematopoietic differentiation. When animal pole tissue is recombined with isolated VMZ cells, an increase in hemoglobin is detected, indicating that the animal pole contains signals that enhance globin expression. A focus of our laboratory is to use these assays to isolate and define factors that participate in ventral axis patterning or mesoderm induction.

SUMMARY

The cellular and environmental regulation of hematopoiesis has been generally conserved throughout vertebrate evolution, although subtle species differences exist. The factors that regulate hematopoietic stem cell homeostasis may closely resemble the inducers of embryonic patterning, rather than the factors that stimulate hematopoietic cell proliferation and differentiation. Comparative study of embryonic hematopoiesis in lower vertebrates can generate testable hypotheses that similar mechanisms occur during hematopoiesis in higher species.

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