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Embryonic Fates for Extraembryonic Lineages: New Perspectives

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ABSTRACT

The prevailing view of the functions of the extraembryonic lineages of the mammalian embryo has been that they serve solely to support its intrauterine development. In recent years, a number of studies have suggested that the extraembryonic mesoderm and visceral endoderm in fact contribute cells to tissues of the developing animal. In this mini-review, we discuss evidence that the yolk sac is an early source of hematopoietic stem and progenitor cells and that the cells of the visceral endoderm, once thought to be segregated solely to the yolk sac, constitute a subpopulation of cells within the developing gut tube and perhaps other endodermal structures. Fascinating questions remain to be addressed and are likely to establish a new paradigm for studying early mammalian development. Understanding the processes that give rise to stem cell populations in development may lead to advances in stem cell therapies and regenerative medicine. J. Cell. Biochem. 107: 586–591, 2009.

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Reciprocal interactions between embryonic and extra-embryonic lineages are critical for establishing the basic body plan of the early mammalian embryo [Baron, 2005; Kwon et al., 2008; Arnold and Robertson, 2009; Rossant and Tam, 2009]. Signals from the visceral endoderm, an extraembryonic tissue, play important roles during gastrulation, a process that gives rise to mesoderm and endoderm [Arnold and Robertson, 2009; Rossant and Tam, 2009]. Molecules secreted by the visceral endoderm (VE) are also involved in the activation of hematopoietic and endothelial cell development [Baron, 2005]. The VE and extraembryonic mesoderm together comprise the yolk sac (YS), a bilaminar membrane that surrounds the developing mammalian embryo. Encased between the two layers of the YS are "blood islands" of primitive erythroid cells (EryP) and surrounding endothelial cells [Haar and Ackerman, 1971], both lineages of mesodermal origin [Lawson et al., 1991; Kinder et al., 2001]. Progenitors of definitive hematopoiesis also form in the YS, at a later stage [Yoder et al., 1997b] as well as intraembryonically, in the para-aortic splanchnopleura (Sp, days 8.5–9.5 [Cumano et al., 1996]) and aorta-gonad-mesonephros region (AGM, days 10.5–11.5 [Medvinsky and Dzierzak, 1996]). The origin of HSCs during embryogenesis has been controversial [Orkin and Zon, 2008; Yoshimoto et al., 2008].

During gastrulation, not only mesoderm but also definitive endoderm (DE) is generated. This tissue is fated to form the epithelial lining of the lungs and digestive tract [Lewis and Tam, 2006]. As we will discuss in this review, until recently, it was believed that the VE and DE remained exclusively segregated during development (Fig. 1).

Dissecting out the possible contribution of extraembryonic lineages to the embryo proper has been difficult, in large part because of the inaccessibility of the mammalian embryo within the uterus. Advances in a number of technologies, including gene targeting, tetraploid re-aggregation, in vitro culture of mouse embryos, lineage-specific fluorescent reporter expression and

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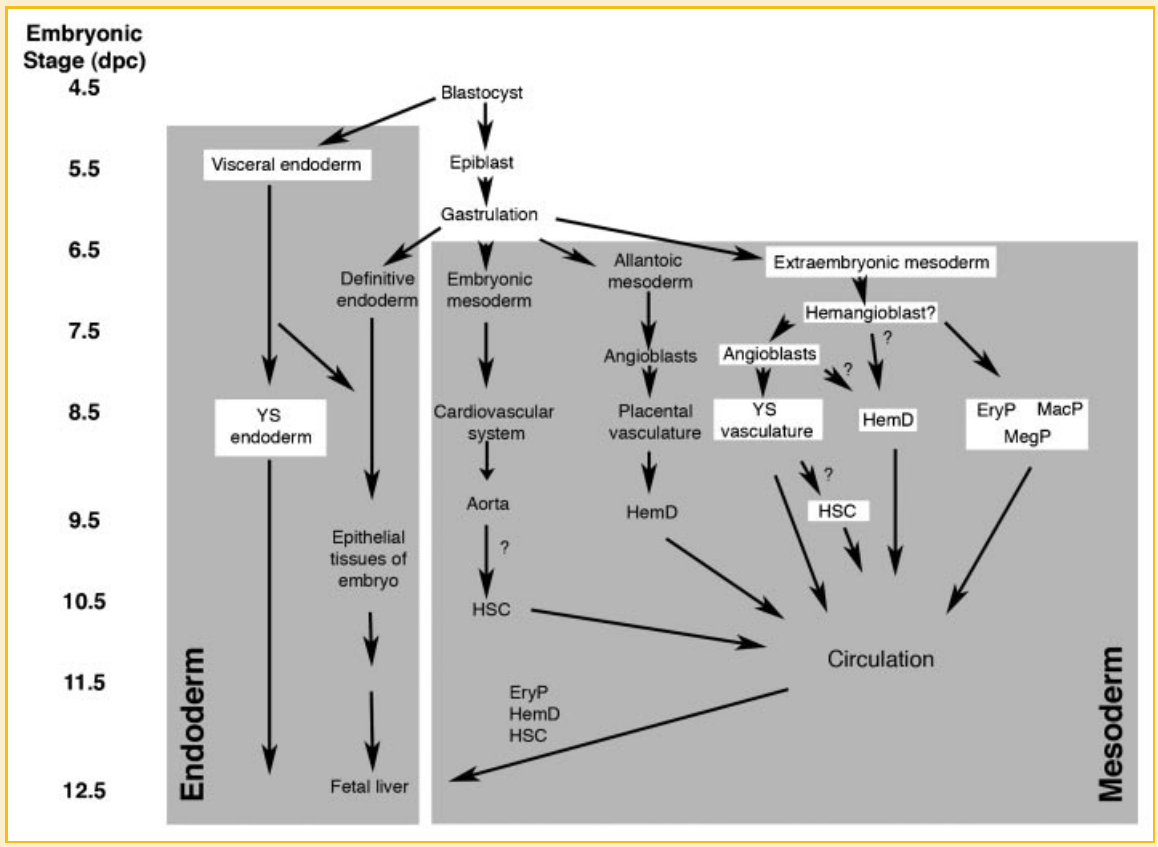

Fig. 1. Development of the endoderm and mesoderm in the mouse embryo. The developmental progression of the endoderm, mesoderm and their derivative cell types is presented (development of the third germ layer, the ectoderm, is not included here). Starting from the blastocyst, the endodermal and mesodermal developmental pathways are depicted, with the extraembryonic tissues boxed in white. HSC, hematopoietic stem cells; HemD, definitive hematopoietic progenitors; EryP, primitive erythroid cells; MacP, primitive macrophages; MegP, primitive megakaryocytes.

genetic labeling of stem cell populations has dramatically enhanced the usefulness of the mouse as a model and, as will be discussed in this mini-review, have provided new insights into the roles of extraembryonic derivatives during development.

YOLK SAC HEMATOPOIESIS: HEMANGIOBLASTS, BLOOD ISLANDS, AND BLOOD BANDS

The close spatial and temporal association in the YS between primitive hematopoiesis, the formation of embryonic blood cells, and vasculogenesis, the formation de novo of blood vessels from endothelial cells (angioblasts), led to the concept of a common progenitor termed the "hemangioblast" [reviewed by Lensch and Daley, 2004; Orkin and Zon, 2008]. Experimental support for such a cell came from studies of differentiating mouse embryonic stem (ES) cells [Choi et al., 1998] and, later, from mouse embryos [Huber et al., 2004]. Hemangioblasts were thought to give rise to "blood islands," clusters of primitive erythroblasts (EryP) surrounded by endothelial cells [reviewed by Ferkowicz and Yoder, 2005]. Two waves of angioblast development have been identified in the YS, one closely associated with, and a second independent of hematopoiesis [Furuta et al., 2006]. The concept of a common hematopoietic and angioblastic cell progenitor for blood islands has been recently questioned on two fronts. First, analysis of mouse chimeras has suggested that blood islands are polyclonal [Ueno and Weissman, 2006]. In these studies, embryos were derived from combinations of blastocysts into which ES cells expressing different fluorescent reporters (either GFP, CFP, RFP, or no reporter) had been injected. A single clonal origin for blood islands would result in islands marked by a single color. A polyclonal origin would result in blood islands marked by a mixture of colors. The latter was observed [Ueno and Weissman, 2006]. This clonal analysis does not completely rule out the existence of hemangioblasts, which are relatively rare cells in the mouse embryo [Huber et al., 2004], but it does suggest that the prevailing view of hematopoietic and vascular development in the yolk sac needs to be revised. Second, the concept of blood islands has been challenged in a careful immunohistochemical analysis of the early mouse YS [Ferkowicz and Yoder, 2005]. In that study, independent blood islands could not be identified. Instead, a ring of CD41(+) cells (hematopoietic progenitors) that gradually circumscribes the proximal YS was observed at the late neural plate stage (E7.75) and termed a "blood band" [Ferkowicz and Yoder, 2005]. It was proposed that this mass or band of primitive erythroid cells and more sparsely distributed endothelial cells is later subdivided by endothelial cell layers [Ferkowicz and Yoder, 2005].

Whether the hematovascular system in the early YS develops in "blood islands" or one [Ferkowicz and Yoder, 2005] or more [Ueno and Weissman, 2006] "blood bands" warrants further investigation.

PRIMITIVE HEMATOPOIESIS IN THE YOLK SAC

The first hematopoietic cells of the embryo do not arise from hematopoietic stem cells (HSC) but from progenitors of the primitive erythroid (EryP) lineage [Wong et al., 1986]. These primitive red blood cells are generated in vast numbers and are rapidly outnumbered by definitive (adult type) erythroid cells once the fetal liver becomes an active hematopoietic organ [Kingsley et al., 2004; Fraser et al., 2007]. Two distinct erythroid progenitors were initially described in the early YS: one gave rise to embryonic globin-expressing, erythropoietin-dependent small colonies, while the second required both erythropoietin and the addition of spleen conditioned medium and expressed adult-type globins [Wong et al., 1986]. The first population is now referred to as primitive erythroid colony-forming cells (EryP-CFC). EryP-CFC are found in the YS during a tight window of development, beginning around E7.5, peaking at E8.25 and then disappearing by E9.0 [Palis et al., 1999]. Primitive erythroid cells are significantly larger than their adult type (or definitive) counterparts, express a unique set of *globin* genes and circulate as nucleated cells for several days after entering the circulation at ~E9.5 [Kingsley et al., 2004; Fraser et al., 2007]. Definitive erythroid (EryD) cells express adult *globin* genes and are released from the microenvironment in which they arise as enucleated reticulocytes. EryP and EryD arise from different populations of nascent mesoderm during gastrulation [Kinder et al., 2001; Baron, 2005]. Contrary to long-held beliefs, EryP do eventually enucleate [Kingsley et al., 2004; Fraser et al., 2007] and the appearance of enucleated EryP is coincident with the elaboration of the fetal liver as a hematopoietic organ [Isern et al., 2008]. EryP continue to mature in the circulation [Fraser et al., 2007], where they up-regulate a series of adhesion molecules [Fraser et al., 2007] before entering the parenchyma of the fetal liver, where they complete their maturation and enucleate [Isern et al., 2008]. Enucleated EryP are then found within the peripheral blood as definitive erythroid cells begin to be produced by the fetal liver and become the predominant erythroid cell type [Fraser et al., 2007].

Megakaryocytes have been isolated from the YS from the same developmental stages in which EryP-CFC are generated and may arise from the same progenitor [Xu et al., 2001; Tober et al., 2007]. These early megakaryocytes are smaller and have a lower DNA content than those found in the fetal liver or adult bone marrow and appear to represent a distinct "primitive megakaryocyte" lineage. A wave of primitive macrophages also appears in the YS, following an initial wave of maternally derived, mature macrophages [Bertrand et al., 2005]. A third wave of YS-derived macrophage progenitors seeds the fetal liver and is considered definitive [Bertrand et al., 2005]. While formation of the various primitive hematopoietic populations is restricted to the YS, progenitors of definitive hematopoietic cells that arise in the YS may contribute to hematopoiesis in the embryo proper.

THE YOLK SAC AS A SOURCE OF DEFINITIVE HEMATOPOIETIC PROGENITORS

In a seminal study, Moore and Metcalf identified the YS as the earliest site of hematopoietic development [Moore and Metcalf, 1970]. Colony-forming cells (CFC) that could be assessed by culture in vitro and expansion within the spleens of recipient mice in vivo were present in the YS but not in the embryo proper at E7.5. Whereas YS tissue cultured alone for 2 days generated large numbers of CFCs, these progenitors were not detected in embryo tissue cultured independently of the YS [Moore and Metcalf, 1970]. By E10.5, after circulation between the YS and embryo is established [McGrath et al., 2003], CFC were detected in the embryo proper [Moore and Metcalf, 1970].

CFC activity was later re-examined in carefully staged embryos [Palis et al., 1999]. The early YS was confirmed as the source of EryP-CFC and a range of CFCs giving rise to definitive erythroid cells, megakaryocytes, granulocytes, macrophages, and mast cells was detected beginning around E8.25 [Palis et al., 1999]. Highly proliferative multilineage progenitors (HPP-CFC) were also found within the YS and, at later stages, in the peripheral blood [Palis et al., 2001]. Cells isolated from the E8.5 YS by flow cytometric sorting on the basis of their expression of VE-cadherin can give rise to definitive erythroid, myeloid and later B lymphoid cells when cultured ex vivo [Fraser et al., 2002].

It has been speculated that the YS is the source of microglial cells, the macrophage-like supporting cells found in the central nervous system [Alliot et al., 1999]. The basis for this claim is that phenotypically similar (F4/80-expressing) cells are initially found in the YS. The authors propose that macrophage progenitors move from the YS into the mesenchyme surrounding the brain rudiment, either through the circulation or by interstitial migration early in development [Alliot et al., 1999]. Lineage tracing experiments will be essential to determine the actual provenance (extraembryonic or embryonic) of microglia within the brain.

THE YOLK SAC VERSUS INTRAEMBRYONIC SOURCES OF HEMATOPOIETIC STEM CELLS

Initially, the YS was proposed as the presumptive site of hematopoietic stem cell (HSC) development, as YS cells injected into irradiated adult mice could reconstitute the hematopoietic systems of the recipients when assessed after 30 days [Moore and Metcalf, 1970]. However, the yolk sac origin of definitive HSCs was challenged when it was discovered that cells from the E10.5 aorta-gonadal-mesonephros (AGM) region of the embryo, but not from the E10.5 YS, possess long-term reconstituting activity in irradiated adult recipients [Muller et al., 1994; Medvinsky and Dzierzak, 1996]. These findings have led to a long-running debate over the site of origin of HSCs in the developing conceptus [Orkin and Zon, 2008; Yoshimoto et al., 2008]. The failure of cells from the YS to engraft in and reconstitute adult mice, it was argued, indicates that these cells do not play an important role in definitive hematopoiesis [Speck et al., 2002]. In fact, E9 YS-derived CD34(+)c-kit(+) cells have the *potential* for engraftment and

long-term repopulation: when transplanted into the livers of newborn mice, these cells repopulate all hematopoietic compartments of the recipient animals and the reconstituted bone marrow can be serially transferred [Yoder and Hiatt, 1997; Yoder et al., 1997a,b]. It appears, therefore, that long term reconstituting potential can be found in both anatomical sites at the same developmental stage, though this is dependent upon the assay used. An intriguing question is whether there are functional differences between these anatomically distinct populations or whether maturation of YS-derived HSCs is required before these cells can contribute to definitive hematopoiesis.

In a set of technically challenging experiments, YS–YS chimeras were created by inserting small pieces of GFP-labeled E8.25 (pre-circulation) YS through a slit in the YS of non-fluorescent embryos and were then cultured ex vivo for over 2 months [Sugiyama et al., 2007]. This approach has revealed low levels of donor YS-derived cells with B cell potential in the AGM region of host embryos [Sugiyama et al., 2007].

An important advantage of the mouse system is the rapidly expanding list of transgenic and null mutant strains. Genetically targeted mutations have been useful in elucidating the contribution of various cell populations to definitive hematopoiesis. A particularly interesting example is the "embryo without a heartbeat" in which the Sodium/Calcium exchanger Ncx1 has been genetically ablated. *Ncx*1-null embryos develop a heart that is grossly normal but contains cardiomyocytes that fail to contract; therefore, the heart fails to beat and circulation never initiates [Koushik et al., 2001]. Analysis of the hematopoietic potential of extra-embryonic and intra-embryonic sites within the mutant embryos revealed active multilineage hematopoiesis in the YS but not within the embryo proper [Lux et al., 2008]. The authors concluded that all primitive and definitive hematopoietic progenitors that emerge before ~E10.25 are of YS origin and that, up to this stage, the YS is the predominant site of hematopoietic progenitors that seed the fetal liver [Lux et al., 2008]. The *Ncx1* mutant mouse has also proven useful in evaluating the placenta as a site of hematopoietic development. The chorion, the allantois, and the placenta (the product of fusion of these extraembryonic tissues) have been demonstrated as sites of definitive hematopoiesis [Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Zeigler et al., 2006; Corbel et al., 2007]. Analysis of *Ncx1* null placentas revealed that definitive hematopoietic progenitors emerge de novo from large vessels within this tissue. Whether the progenitors that arise in the YS [Lux et al., 2008] or placenta [Rhodes et al., 2008] are functional HSCs, as defined by hematopoietic reconstitution following transplantation, remains unknown.

Little is known about the mechanisms underlying HSC migration to fetal tissues such as the liver. Homing of HSCs to and engraftment within the adult bone marrow is guided by molecules such as stromal derived factor-1α (SDF-1α) and its receptor, CXCR4, c-kit, CD44, and β1 integrin [Papayannopoulou, 2003]. Intracellular pathways downstream from these molecules are integrated by Rho GTPases such as the Rac proteins. Rac1-deficient hematopoietic stem and progenitor cells (HSPCs) display defects in engraftment following transplantation [Gu et al., 2003]. However, if *Rac1* is deleted *after* the cells have engrafted, steady-state hematopoiesis is normal [Gu et al., 2003]. Conditional ablation of *Rac1* in the hematopoietic compartment indicated that *Rac1* is not required for development of HSPCs in the YS but is required for intraembryonic hematopoiesis [Ghiaur et al., 2008]. *Rac1*-deleted HSPCs in the YS and in the circulation showed altered migration properties in vitro [Ghiaur et al., 2008]. These results suggest that the YS (and perhaps the placenta, not examined in this study) is the source of the first HSCs and that their migration into the embryo is required for normal hematopoietic development [Baron, 2008]. They do not, however, formally exclude that HSCs are unable to form de novo within the AGM region and other intraembryonic sites within *Rac1* deficient embryos, a scenario that would be consistent with both extra- and intra-embryonic locations for the emergence of HSCs [Baron, 2008].

In a fate mapping study, an *estrogen receptor-Cre* fusion gene was knocked into the *Runx1* locus to generate Runx1$^{mER\text{-}Cre\text{-}mEr/+}$ mice [Samokhvalov et al., 2007]. When the latter were crossed with Rosa26R$^{LacZ/+}$ animals, doubly transgenic embryos could be genetically labeled upon induction by the estrogen analog tamoxifen. The authors argued that cells labeled within a 24 h period and found only within the YS at E7.5 give rise to the full range of hematopoietic lineages in the adult [Samokhvalov et al., 2007]. While this system is powerful, the study raised a number of questions. If tamoxifen were to remain within the embryo for longer than 24 h, other *Runx1*-expressing cells, possibly not restricted to the YS, would also be labeled and their progeny would be detected later in life. In addition, at E7.5, *Runx1* is clearly expressed in the chorion and allantois prior to their fusion and *Runx1*-expressing cells in these tissues have definitive hematopoietic potential [Zeigler et al., 2006]. Another significant issue is that *Runx1* haploinsufficiency (which would accompany knock-in of the *estrogen receptor-Cre* fusion gene) results in changes in the temporal and spatial distribution of HSCs within the embryo and YS [Cai et al., 2000]. Therefore, whether the first HSCs arise locally within the AGM region or migrate from the YS [Moore and Metcalf, 1970; Kumaravelu et al., 2002] remains unresolved.

THE VISCERAL ENDODERM

Two types of endoderm arise during embryogenesis. The first, visceral endoderm (VE), is specified during the blastocyst stage [Zaret, 2002]. Later, the conceptus develops into a cup-shaped structure in which the VE surrounds the epiblast, which will form the body of the embryo and will give rise to extraembryonic mesoderm [Arnold and Robertson, 2009; Rossant and Tam, 2009]. Definitive endoderm (DE) is generated during gastrulation. Until recently, cells of the VE were believed to remain confined to the YS [Hogan, 2006 #13318]. It had been the conventional wisdom that, during gastrulation, the DE expanded and displaced the VE more proximally, toward extraembryonic regions [Hogan and Zaret, 2002], covering the entire epiblast except for a small region overlying the posterior streak [Arnold and Robertson, 2009; Rossant and Tam, 2009]. In contrast, the DE formed the epithelial lining of the respiratory and digestive tracts, including the lungs, intestines, liver and pancreas, with no contribution from the VE [Hogan and Zaret, 2002]. However, in an elegant cell fate analysis, fluorescent

reporters expressed under the control of lineage-specific regulatory elements revealed that cells of the VE are not strictly segregated from the DE but instead persist in the layer of DE [Kwon et al., 2008]. Thus, the gut endoderm appears to be of dual origin, DE and VE. Whether the VE cells survive to integrate into the tissues of the embryo and give rise to endodermal progenitors found within the adult has yet to be determined [Kwon et al., 2008]. Given the well described secretory functions of the VE [Rossant, 1986], the discovery that some VE-derived cells are organized around key midline signaling centers involved in patterning the developing epiblast (including the primitive streak, node, and head process) [Kwon et al., 2008] is particularly intriguing.

CONCLUDING REMARKS

While it has generally been agreed that the developing fetal liver is seeded by migrating HSCs and serves as a niche for their differentiation along most blood lineages, the origin of HSCs during ontogeny has been in dispute for the past 15 years. A number of studies, reviewed above, provide some support for the physiological relevance for definitive hematopoiesis of the multipotent progenitors formed in the YS. It is not yet entirely clear how some of the conflicting data and conclusions reviewed here can be reconciled. However, it will be important to explore the differences between HSC from the YS *versus* the embryo proper, as they may offer insights into mechanisms for expanding HSC ex vivo, an advance that would be of great utility for transplantation therapy and regenerative medicine. The pre- and peri-gastrulation regulatory functions of another extraembryonic tissue, the VE, have been appreciated for some time. It also now appears that, for at least part of its development and possibly throughout postnatal life, the gut contains cells derived from the VE. The same may be true for the lung. Do endodermal cells of VE versus DE origin have distinct functions? Many questions remain unanswered and the paradigms of today will no doubt be challenged by technical innovations to be developed in the future.

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