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Primitive and definitive erythropoiesis in the yolk sac: a bird's eye view

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ABSTRACT The yolk sac is the sole niche and source of cells for primitive erythropoiesis from E1 to E5 of chicken development. It is also the main niche and source of cells for early definitive erythropoiesis from E5 to E12. A transition occurs during late embryonic development, after which the bone marrow becomes the major niche and intraembryonically-derived cells the major source. How the yolk sac is involved in these three phases of erythropoiesis is discussed in this review. Prior to the establishment of circulation at E2, specification of primitive erythrocytes is discussed in relation to that of two other cell types formed in the extraembryonic mesoderm, namely the smooth muscle and endothelial cells. Concepts of blood island, hemangioblast and hemogenic endothelium are also discussed. It is concluded that the chick embryo remains a powerful model for studying developmental hematopoiesis and erythropoiesis.

KEY WORDS: *chicken, primitive erythropoiesis, definitive erythropoiesis, hematopoiesis, yolk sac*

Introduction

Studies on chicken hematopoietic development have been instrumental in the establishment of several key concepts in the field, including B lymphocytes, hematopoietic stem cells, hemangioblasts and hemogenic endothelium. Research focus in recent years has shifted heavily toward genetic and molecular based investigation, leading to a bias against the use of avian models. The wealth of published literature on descriptive and experimental analyses of chicken hematopoiesis, however, is still unsurpassed by any other model organism. The completion of chicken genome (International Chicken Genome Sequencing Consortium, 2004) has made molecular analysis relatively straightforward and genetic analysis possible. In addition to superb spatial and temporal resolution in developmental studies, the chick model serves as an evolutionary link between mammals and lower vertebrates. Here I will provide an embryological overview of chicken extraembryonic hematopoietic development, and focus more detailed discussion on the involvement of the yolk sac in the generation of primitive and definitive red blood cells. Readers interested in other related aspects of chicken hematopoietic development may find relevant details in articles and monographs for general description of hematopoiesis (Dieterlen-Lievre and Le Douarin, 2004; Jaffredo *et al.*, 2003; Le Douarin, 1978; Metcalf and Moore, 1971; Romanoff, 1960), molecular regulation of hematopoiesis (Bollerot *et al.*, 2005; Minko *et al.*, 2003; Siatskas and Boyd, 2000), hematological description of different blood lineages (Lucas and Jamroz, 1961), early vascular morphogenesis (Drake, 2003; Drake *et al.*, 1998; Drake *et al.*, 2000) and developmental and adult immune systems (Davison *et al.*, 2008).

Yolk sac, amnion, chorion and allantois

Extraembryonic tissues in developing chick embryo contain the following four components: yolk sac, amnion, chorion and allantois (Fig. 1 A,B). The term "extraembryonic", however, is somewhat a misnomer, as there is no clear anatomical or molecular demarcation to separate the embryonic from the extraembryonic tissues during the first few days of development, and at later stages both are integral components of the developing embryo. This is especially so for the hematopoietic system after the establishment of circulation by the end of embryonic day 2 (E2). Furthermore, the yolk sac, the most prominent component of the extraembryonic tissues, is withdrawn into the abdomen after hatching and is technically embryonic. The allantois, formed near the tail bud at the junction between embryonic and extraembryonic tissues, has contributions from both. Nevertheless, this review will follow the conventional view of these tissues as extraembryonic.

Before formation of the amnion, chorion and allantois, the yolk sac is the only extraembryonic tissue (Fig. 1 C,D). The amnion

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and chorion are derived from yolk sac somatopleure. Expansion of extraembryonic coelom separates these two somatopleural tissues from the splanchnopleural tissue, which becomes the later yolk sac. The amnion encloses the developing embryo by the end of E3 (Adamstone, 1948; Lillie, 1919; Romanoff, 1960; Wu *et al.*, 2001). The remainder of the extraembryonic somatopleure becomes the chorion. The allantois grows rapidly from a small out-pocket of hindgut anlage during E3, and from E5 it starts to fuse with the chorion into chorioallantois (DeFouw *et al.*, 1989; Leeson and Leeson, 1963; Lillie, 1919; Melkonian *et al.*, 2002; Romanoff, 1960; Steinmetz, 1930). The formation of chorioallantois involves only the distal wall of the allantois. Its proximal wall starts to fuse with the amnion from E7 (Romanoff, 1960; Steinmetz, 1930) and with the yolk sac during the last few days of embryonic development (Romanoff, 1960). From the perspective of germ layer contribution, the allantois contains endoderm and mesoderm cells, the amnion and chorion contain ectoderm and mesoderm cells, and the yolk sac contains all three germ layers at early stages and mesoderm and endoderm cells after the expansion of the extraembryonic coelom.

Area pellucida, area opaca and area vasculosa

For the convenience of embryological description and manipulation, early chick blastoderm is separated into area pellucida and area opaca based on the opaque appearance of endoderm cells in the outer region with abundant intracellular yolk granules and attached acellular yolk (Bellairs, 1958; Bellairs, 1963; Bellairs, 1964) (Fig. 1 C,D). This does not, however, correspond to the traditional division of intraembryonic and extraembryonic tissues, because a significant part of the area pellucida contributes also to extraembryonic tissues (Fig. 1D). Depending on the extent of mesoderm cell invasion, the area opaca is further divided into the area vasculosa, area vitellina and margin of overgrowth (Bellairs, 1963). The margin of overgrowth, located at the extreme peripheral region, contains a special type of ectoderm cells responsible for maintaining epiblast tension and for the epiboly process that eventually encloses the entire yolk (Bellairs, 1963; Bellairs *et al.*, 1967; New, 1959). The area vitellina is the region of the yolk sac that does not yet contain mesoderm cells. The area vasculosa, the place where yolk sac vasculogenesis and hematopoiesis take place, is marked laterally by the extent of extraembryonic mesoderm invasion (Fig. 1 B,D). Medially, the area vasculosa is continuous with vascular regions in the area pellucida. The area vasculosa expands laterally to replace the area vitellina (Bellairs, 1963; Romanoff, 1960). When circulation starts at E2 (Fig. 1 C,D), the area vasculosa occupies only a very small part of the entire yolk sac, whereas the margin of overgrowth has already expanded to the equatorial region. By E5 (Fig. 1 A,B), the area vasculosa reaches the equator and the area vitellina has covered most of the yolk. By E15, the area vasculosa covers the entire yolk and the area vitellina disappears. The expansion of the area vitellina and area vasculosa shows noticeable variations in relationship both to each other and to the growth of the embryo proper based on the Hamburger and Hamilton staging criteria (Hamburger and Hamilton, 1992), suggesting a need in the future to establish an independent staging system for the extraembryonic tissues. As yolk sac erythropoiesis takes place exclusively in the mesoderm lineage, only the area vasculosa part of the yolk sac will be discussed in this review. Early yolk sac erythropoiesis occurs in the extraembryonic mesoderm before the expansion of the extraembryonic coelom and the invasion of the allantois, and thus in the context of all three germ layers. Late yolk sac erythropoiesis takes place in splanchnic mesoderm-derived tissues adjacent to the yolk sac endoderm.

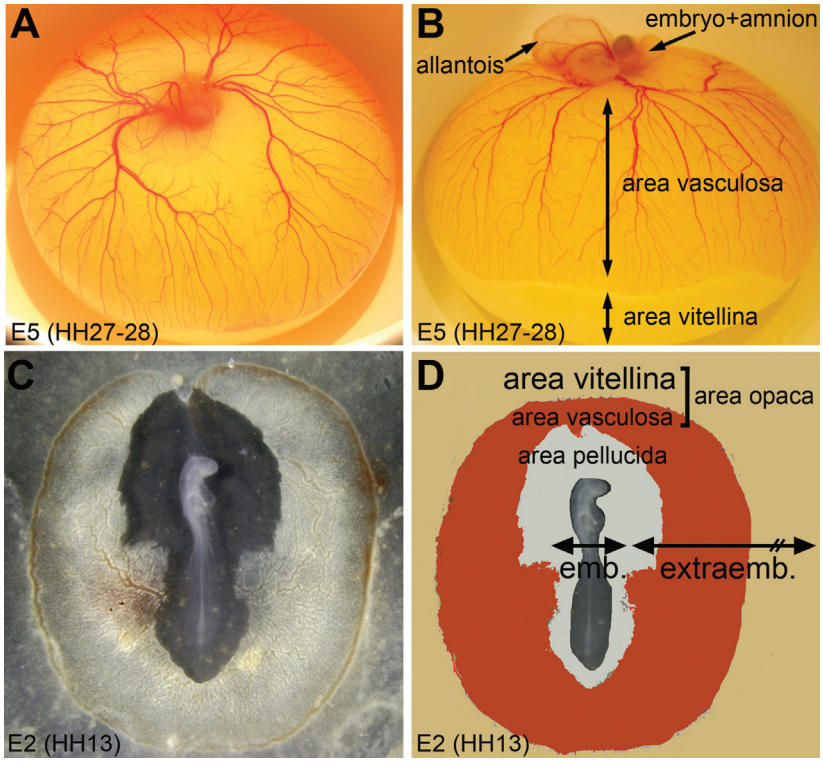

Fig. 1. General organization of chicken extraembryonic tissues. (A,B) *E5 embryo.* **(C,D)** *E2 embryo.* **(A)** *Top view of an E5 embryo before breaking the vitelline membrane and chorion.* **(B)** *Top-side view after partial breaking of the vitelline membrane and chorion to reveal the embryo proper (with the amnion) and allantois. At this stage, the area vasculosa has reached the equator and the area vitellina has reached the opposite pole (not seen here).* **(C)** *Top view of an E2 (HH13) embryo, showing the area pellucida and area vasculosa. Most of the area vitellina is not included here.* **(D)** *Schematic diagram of (C).*

Extraembryonic mesoderm and ventral mesoderm

All mesoderm cells are generated from the primitive streak in the area pellucida (timeline in Figure 4). The most lateral extraembryonic mesoderm cells are derived from early mesoderm cells generated from stage HH2 onward in the elongating primitive streak. Fates of mesoderm precursors in the primitive streak vary depending on the stages. Generally speaking, posterior part (1/2 to 1/3) of the primitive streak continues to give rise to extraembryonic fated mesoderm from HH2 to at least HH9 (Murray, 1932; Nakazawa *et al.*, 2006; Nocolet, 1970; Peebles, 1898; Psychoyos and Stern, 1996; Rawles, 1936; Rosenquist, 1966; Rudnick, 1938;

Sawada and Aoyama, 1999; Schoenwolf *et al.*, 1992; Spratt, 1942; Spratt, 1946; Spratt, 1947; Spratt and Haas, 1965). Relative to more axially located mesoderm lineages (the axial, paraxial, intermediate and lateral plate mesoderm), extraembryonic mesoderm precursors are of the ventralmost fate and populate the lateralmost regions of the developing embryo. The extraembryonic mesoderm covers a wide area and has its own dorsal/ventral and anterior/posterior patterning processes. The chick embryo during first few days of its development takes the shape of a relatively flat sheet (Fig. 2), and the dorsal/ventral axis of the extraembryonic mesoderm is reflected in the medial/lateral positional difference relative to the embryo proper. Its anterior/posterior axis is similar to that in the embryo proper and reflects the extent of extraembryonic mesoderm migration from the posterior end of the primitive streak to the anterior-most position rostral to the embryonic forebrain. This aspect has not received much investigation so far, although early work by Sabin provided some descriptions of anterior/posterior positional difference in early phases of primitive hematopoiesis (Sabin, 1920). Within the extraembryonic mesoderm, the axis from the extraembryonic ectoderm to the extraembryonic endoderm is sometimes also referred to as dorsal/ventral. To avoid confusion, mesoderm positional difference along this axis is discussed here with specific reference to the ectoderm, endoderm or coelomic cavity.

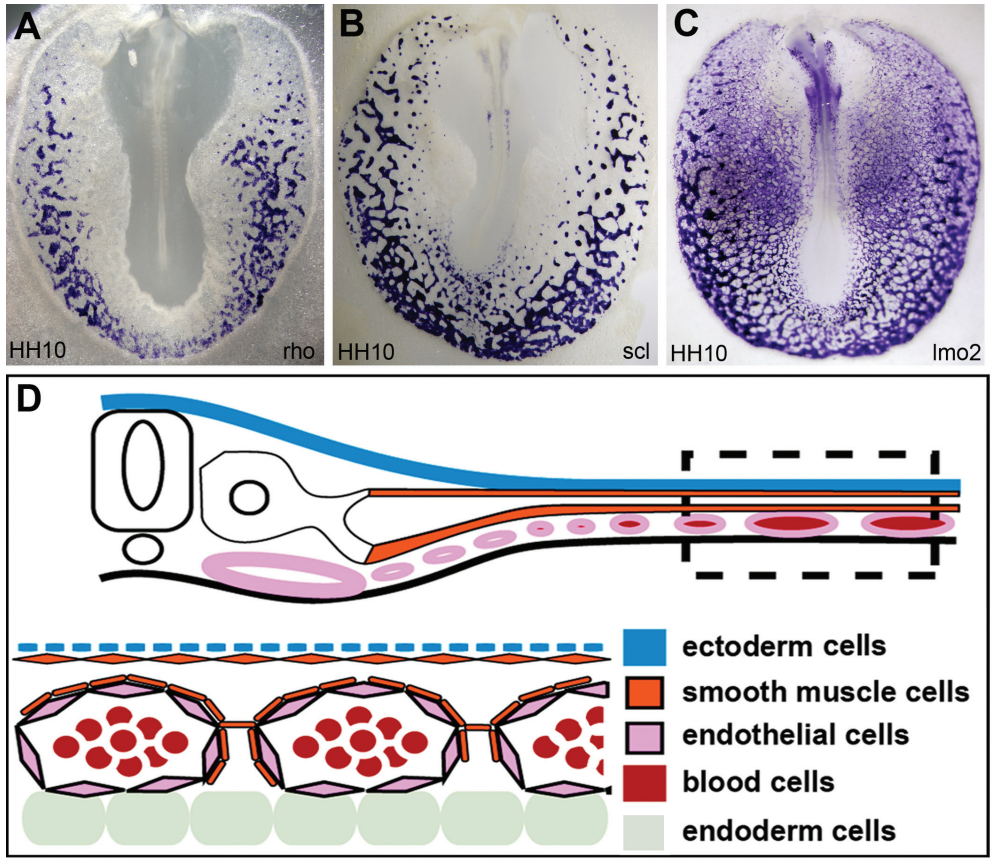

Fig. 2. Primitive erythropoiesis in the yolk sac. (A) rho *globin expression at HH10, revealed using a* rho *intron-specific probe.* **(B)** scl *expression at HH10, marking the blood precursors at this stage.* **(C)** lmo2 *expression at HH10, marking both the developing vasculature and blood cells.* **(D)** *Schematic diagram of an E2 embryo section, showing primitive blood cells in relationship to the ectoderm, endoderm and other extraembryonic mesoderm cell types.*

Ventral mesoderm lineages

During and after the migration to populate extraembryonic territories, mesoderm cells differentiate into three main lineages: blood, endothelial and smooth muscle cells (Fig. 2) (Shin *et al.*, 2009). Yolk sac splanchnic mesoderm contains all three lineages (Fig. 2D). Yolk sac somatic mesoderm contains mainly smooth muscle cells that line the extraembryonic ectoderm (Adamstone, 1948; Oppenheim, 1966; Pierce, 1933; Romanoff, 1960; Shin *et al.*, 2009; Wu *et al.*, 2001) (Fig. 2D). Small and infrequent blood islands with differentiated blood and endothelial cells have also been observed in the extraembryonic somatopleure in our analyses (unpublished data) (Alev *et al.*, 2010), suggesting that blood and endothelial lineages do not segregate completely into the splanchnopleure during early extraembryonic mesoderm patterning. Among these three lineages, progenitors for the smooth muscle cells, contributing to both the somatopleure and splanchnopleure, separate first from the other two soon after their ingression through the primitive streak (Shin *et al.*, 2009), followed by the separation of blood and endothelial cells within the blood island cell population (Nakazawa *et al.*, 2006). Sabin reported a similar observation that the cells contributing to exocoelomic walls separate first from the angioblasts (blood island cells) (Sabin, 1917; Sabin, 1920). The splanchnic wall of the extraembryonic coelom is the source of cells for the vascular smooth muscle layer during vasculogenesis, and after formation of the vascular network, vascular smooth muscle layer is in direct contact with the extraembryonic coelomic cavity. This general organization of somatopleural and splanchnopleural tissues in the yolk sac is supported by ultrastructure studies (Kessel and Fabian, 1985; Murphy and Carlson, 1978).

Blood island

Progenitor cells for blood and endothelial cells form aggregates. This is the earliest morphological sign of differentiation in the extraembryonic mesoderm. Different terms have been used to describe these aggregates, including blood islands, angioblasts and hemangioblasts. Wolff (1764 Theorie von der generation) is generally credited for the earliest description of the blood islands. Initially called “substanzinseln” by Wolff and “Island of Wolff” in English literature, some confusion regarding the connotation of the term “blood island” arose in early 20th century [summarized in

(Adelmann, 1966; Ruckert, 1906)]. This confusion, still outstanding today, is due to three main observations already apparent to embryologists a century ago (Ruckert, 1906): 1) prior to circulation, aggregates can be observed in the area vasculosa that are associated with both vasculogenesis and hematopoiesis; 2) aggregates can be observed both before and after the appearance of vascular endothelial cells; and 3) aggregates observed before the appearance of vascular endothelial cells are associated more with the territory of vasculogenesis than with that of hematopoiesis. These seemingly contradictory observations, although not so with current knowledge, led Sabin to call early blood islands prior to the appearance of vascular endothelial cells as angioblasts and later blood islands inside the vasculature as true blood islands (Sabin, 1920). Early blood islands, Sabin's angioblasts, were referred to as hemangioblasts by Murray (Murray, 1932). Each hemangioblast, capable of generating both blood and endothelial cells but not necessarily giving rise to both, was defined by Murray as one blood island aggregate, containing many cells. This definition is different from the current use of hemangioblast as a single cell capable of generating blood and endothelial cells after division(s) (Choi *et al.*, 1998; Huber *et al.*, 2004; Park *et al.*, 2005; Ueno and Weissman, 2006; Vogeli *et al.*, 2006). Angioblasts of Sabin are not often used in current literature and hemangioblasts of Murray currently have a strong connotation of cell differentiation potentials. This review uses the term blood island(s) to describe the aggregate(s) prior to the formation of vasculature, blood cell aggregate(s) to describe Sabin's blood island(s) inside vessel lumen prior to their separation into individual blood cells, and hemangioblasts, in the sense that each is a single cell, only when referring to the bi-potential nature of these early blood island cells.

Larger blood islands, constituting more cells in a given blood island and with more cells percentage-wise contributing to the blood lineage, are present in more lateral regions of the area vasculosa. Progressively smaller blood islands are observed medially (Fig. 2). Blood islands formed in extraembryonic part of the area pellucida and in the intraembryonic lateral plate are very small, containing a few cells and only giving rise to endothelial cells. Thus during primitive erythropoiesis, the area vasculosa can be divided into hemogenic and non-hemogenic regions. Blood islands in the hemogenic region generate both primitive blood and endothelial cells and those in the non-hemogenic region generate only endothelial cells (Nakazawa *et al.*, 2006; Ruckert, 1906; Sabin, 1920). The transition between hemogenic and non-hemogenic regions is gradual and not clearly demarcated morphologically or molecularly. This is also true for the transition between the non-hemogenic extraembryonic region and the intraembryonic lateral plate vessels. During primitive hematopoiesis, erythrocyte formation has always been observed in association with endothelial cell formation in our studies, whereas endothelial cells often form from small blood islands with no accompanying erythropoiesis.

Primitive erythrocyte differentiation

Blood islands first appear at around stage HH6 in a U or V shaped area marking the posterior and lateral extents of extraembryonic mesoderm expansion. The earliest erythropoietic differentiation marked by hemoglobin gene expression occurs at stage HH7 (0-1 somite stage) (Nakazawa *et al.*, 2006). Molecularly, however, extraembryonic mesoderm differentiation starts as soon as they are generated from the posterior primitive streak. The earliest appearance of extraembryonic coelom varies from HH6 to HH8. Hemoglobin expression appears initially only in the most

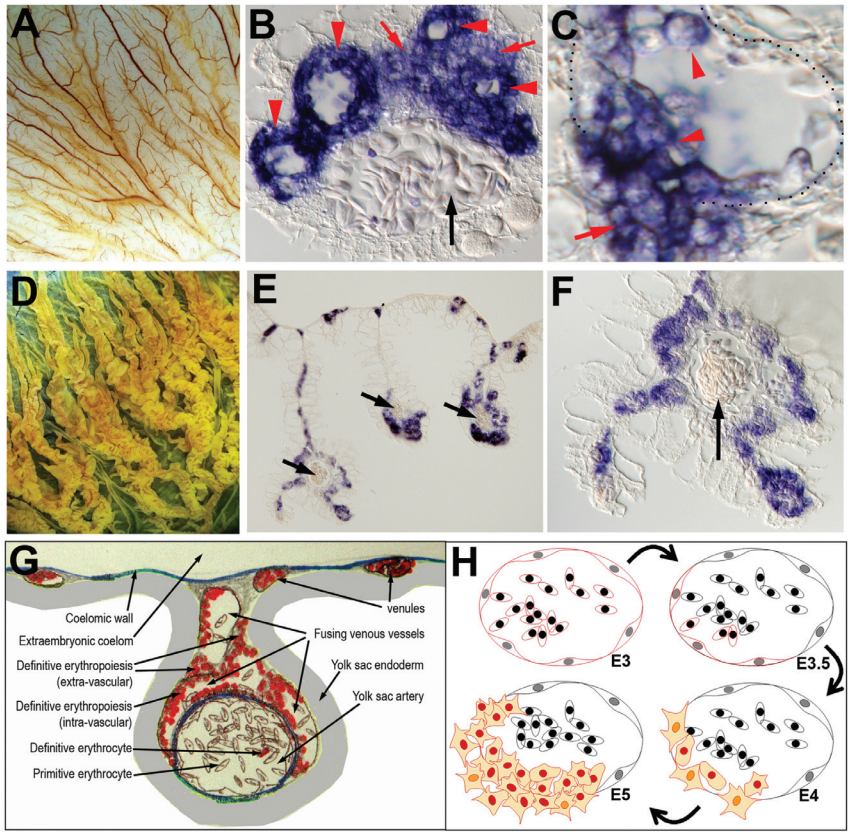

Fig. 3. Definitive erythropoiesis in the yolk sac. (A) *View of a region of E5 yolk sac from the ectoderm side.* **(B,C)** *Section of E5 (B) or E4 (C) yolk sac after* in situ *using a* betaA *globin-specific locked nucleic acid (LNA)-based probe, showing active erythropoiesis in association with venous vessels. Black arrow: artery. Red arrows: extra-vascular clusters. Red arrowheads: intra-vascular clusters.* **(D)** *View of a region of E10 yolk sac from the endoderm side.* **(E,F)** *Section of E10 yolk sac after* in situ *using the* betaA *LNA probe, showing active erythropoiesis in association with venous vessels. More intra-vascular clusters are seen at this stage. Arrows: artery.* **(G)** *Schematic drawing of E5 yolk sac section.* **(H)** *A model for how early definitive erythrocytes are generated in the yolk sac. Two possible sources, as discussed in the text, are combined here in this model. At E3, both primitive erythrocytes and vascular endothelial cells retain some hematopoietic potential. At E3.5, this potential is lost in most primitive erythrocytes and endothelial cells, but is retained or enhanced in a small percentage of both cell types. At E4, dedifferentiation of these hematopoietic cells occurs, leading to formation of hematopoietic cell clusters, and at slightly later stage (E4.5-5), of erythropoietic clusters. Some of these hematopoietic cells may give rise to other blood lineages or stem/progenitor cells (marked with orange nucleus in E4 and E5).*

peripheral blood islands, reflecting progressive phases of differentiation (Fig. 2 A-C). Although it is considered that more centrally located cells in a given blood island differentiate into blood whereas outer cells into endothelial cells, details of morphological differentiation at the cellular level are so far unclear. Within a given blood island, hemoglobin gene expression precedes morphological distinction between blood and endothelial cells (Nakazawa *et al.*, 2006). Although cell divisions occur in undifferentiated extraembryonic mesoderm cells and subsequently in blood island cells and later differentiated mesoderm lineages, the cell fate choice between blood and endothelial lineages among blood island cells is made largely in the context of cell population, instead of progenies of cell divisions (Weng *et al.*, 2007), suggesting that hemangioblasts as currently defined do not play a major role during primitive blood and vascular formation. After the differentiation of blood island cells into blood and endothelial lineages, endothelial cells from individual blood islands anastomose to form primary vascular plexus. By stage HH10 (10 somites), before the initiation of circulation at stage HH12-13, a well-formed vascular network is recognizable in the area vasculosa (Fig. 1C). In the hemogenic region of the area vasculosa, blood cells at stage HH10 are still observed as clusters, the blood islands by Sabin's definition. Individual blood cells have smoother surface than at earlier stages, yet they still adhere to each other and to the endothelial wall. By stage HH12-13, when the circulation starts, most blood cells in well established vascular channels have lost contacts to each other and to the endothelium, and become free circulating cells (Fig. 4). More medially located blood islands at this stage still have an "immature" morphology and are not connected to the circulation, reflecting the fact that they are generated later than laterally located ones.

Primitive *vs.* definitive erythrocytes

The shift from primitive to definitive erythropoiesis is considered to start at E5 of chicken development. This is based on studies of dynamic changes in hemoglobin composition and erythrocyte morphology in circulating blood. Physiological and molecular mechanisms regulating the timing of this shift are not clear, although change in tissue oxygen levels has been hypothesized as one possible cause (Baumann and Meuer, 1992; Baumann *et al.*, 1983). Since the first report of partial beta globin peptide sequences from normal and sickle human blood cells (Ingram, 1956), efforts were made throughout the sixties and seventies to understand developmental changes in chicken hemoglobin heterogeneity at the protein level (Beaupain *et al.*, 1979; Brown and Ingram, 1974; Bruns and Ingram, 1973; Fraser, 1961; Hashimoto and Wilt, 1966; Manwell *et al.*, 1966; Manwell *et al.*, 1963; Saha, 1964; Saha and Ghosh, 1965; Shimizu, 1972; Simons, 1966; van der Helm and Huisman, 1958; Wilt, 1962; Wilt, 1967). This was followed by the elucidation of chicken hemoglobin genes and genomic organization in the early eighties (Dodgson and Engel, 1983; Dodgson *et al.*, 1981; Dodgson *et al.*, 1983; Dodgson *et al.*, 1979; Dolan *et al.*, 1983; Dolan *et al.*, 1981; Engel and Dodgson, 1980; Engel *et al.*, 1983; Reitman *et al.*, 1993; Villeponteau *et al.*, 1982; Villeponteau and Martinson, 1981) and of developmental changes in hemoglobin transcript profiles in the ninties (Mason *et al.*, 1995; Minie *et al.*, 1992). These studies, together with more recent genomic analysis, can be summarized as follows. There are seven hemoglobin genes in chickens, three of alpha type (pi-alphaD-alphaA) and four of beta type (rho-betaH-betaA-epsilon) in alpha and beta loci, respectively. Both chicken alpha and beta loci are orthologous to eutherian (true mammals) counterparts (Patel *et al.*, 2008). pi is orthologous to human zeta/pseudo-zeta, alphaD to human mu (also called pseudo-alpha2 or alphaD) and alphaA to human pseudo-alpha1/alpha2/alpha1/theta. Individual beta hemoglobin genes in chickens and mammals arose by independent duplications and do not form orthologous groups, although their ancestral beta genes before duplication are considered orthologous (Alev *et al.*, 2009; Cooper *et al.*, 2006; Goh *et al.*, 2005; Patel *et al.*, 2008). Primitive erythrocytes in chickens have high pi, rho and epsilon hemoglobin proteins, and definitive erythrocytes have high alphaD, alphaA and betaA. All three alpha globins, however, are present in both primitive and definitive erythrocytes. betaA, in addition to being the major beta globin in definitive erythrocytes, is also weakly present in primitive erythrocytes (Alev *et al.*, 2008). betaH is not detected in primitive erythrocytes and is very low in definitive erythrocytes (Alev *et al.*, 2008; Mason *et al.*, 1995). Analyses on developmental changes of hemoglobin heterogeneity suggest that definitive erythrocytes appear in circulation at E5 and start to constitute the majority of circulating erythrocytes by the end of E7 (Fig. 4). Changes in hemoglobin subtypes have so far only been analyzed with relatively large numbers of blood cells. It is therefore unclear whether changes in hemoglobin transcript and protein heterogeneity can occur at single cell level, either in differentiated erythrocytes or in erythropoietic cells of either lineage.

Both primitive and definitive erythrocytes are nucleated. Mature primitive and definitive erythrocytes have distinct oval shapes measuring on average 8 by 12.7 microns for the former and 7 by 10.3 microns for the latter (Romanoff, 1960). Both lineages progress through several maturation stages, from the most immature megaloblast/hemocytoblast, through proerythroblast, basophilic erythroblast, polychromatophilic erythroblast and reticulocyte, to the final mature erythrocyte (Bruns and Ingram, 1973; Dantschakoff, 1908; Dawson, 1936; Edmonds, 1966; Forkner, 1929; Lemez, 1964; Lucas and Jamroz, 1961; Romanoff, 1960; Small and Davies, 1972; Sugiyama, 1926). These distinctions have been made based on a number of criteria, including general cell and nuclear morphology, histological staining, ratio of nuclei and cell sizes and intracellular organelle and microtubule abundance and distribution. They are all referred to here as erythrocytes based on the fact that even the most immature megalobalsts/hemocytoblasts have initiated hemoglobin expression. These studies on erythrocyte morphological changes revealed a similar picture to that derived from hemoglobin heterogeneity studies. Definitive lineage cells appear in circulation at E5 and overtake primitive lineage cells percentage-wise by the end of E7. Morphological studies also showed that by the time circulation starts at E2, primitive erythrocytes are still at the "proerythroblast" stage with large and round shape. By the end of E3, oval morphology of polychromatophilic erythroblasts takes over, which last until E6, when they develop first into reticulocytes and finally into mature erythrocytes at the beginning of E9 with a complete loss of cytoplasmic ribosomes. Therefore, by the time definitive lineage appears in circulation in the middle of E5 and takes over as the major lineage by the end of E7, the primitive erythrocytes have not completed its maturation process (Fig. 4). Maturation of definitive

erythrocytes follows similar progression of stages to that of the primitive lineage, with a notable difference in that the definitive lineage cells do not mature synchronously, due to the fact that new definitive cells are continuously being generated as the embryo grows.

Transition from primitive to definitive wave

Before definitive erythrocytes enter circulation in the middle of E5, their progenitor cells have been reported to be associated with venous vessels in the yolk sac from the beginning of E4 (Dantschakoff, 1908; Dantschakoff, 1909; Sabin, 1917). This observation is supported by our recent molecular studies (Nagai and Sheng, 2008). Chick/quail and chick/chick chimera studies (Beaupain *et al.*, 1979; Dieterlen-Lievre, 1975; Dieterlen-Lievre *et al.*, 1976; Lassila *et al.*, 1978; Lassila *et al.*, 1982; Martin *et al.*, 1978) and chick/chick parabiotic studies (Metcalf and Moore, 1971; Moore and Owen, 1965; Moore and Owen, 1967) indicated that, at least during the first few days of definitive erythrocyte generation, the source of cells is mainly yolk sac-derived. At about the same time, if not earlier, intraembryonic hematopoietic cells have been observed in association with the dorsal aorta (Dieterlen-Lievre and Martin, 1981; Jaffredo *et al.*, 2003; Jaffredo *et al.*, 2005; Jaffredo *et al.*, 2000; Jaffredo *et al.*, 1998; Jordan, 1916; Nagai and Sheng, 2008; Olah *et al.*, 1988; Pardanaud *et al.*, 1996). A number of questions can be asked, for instance, about whether dorsal aorta-derived hematopoietic cells undergo definitive erythropoiesis either in circulation or in the yolk sac niche; whether yolk sac-derived hematopoietic cells can find their way to the dorsal aorta; and what is the exact type of cells from the yolk sac that contribute to early definitive erythropoiesis. Chimera studies suggested that even at E5-6, though making minor contributions, intraembryonically-derived cells can generate definitive erythrocytes. Dorsal aorta-derived hematopoietic cells, currently considered to be the only intraembryonic hematopoietic source, may be involved in this process. Our molecular analyses with hematopoietic markers support the notion that hematopoietic cells associated with dorsal aorta are derived from dorsal aorta endothelium (Nagai and Sheng, 2008). This is in agreement with data from cell labeling and EM analyses (Jaffredo *et al.*, 1998). Erythropoiesis, however, is not observed at this early period in either intra-vascular or peri-vascular hematopoietic cells associated with the dorsal aorta (Geerts *et al.*, 1993) (H. Nagai and G. Sheng, unpublished data). Our observations also suggest that during this period, most yolk sac vasculature-associated hematopoietic cells undergo definitive erythropoiesis *in situ* (Nagai and Sheng, 2008). Dantschakoff distinguished two hematopoietic populations in the yolk sac, the intra-vascular erythropoietic cells and extra-vascular leucopoietic cells (Dantschakoff, 1908; Dantschakoff, 1909). Our observations, however, suggest that at least for erythropoiesis, it can occur both intra- and extra-vascularly (Nagai and Sheng, 2008) (Fig. 3 A-C,G,H). This is supported by a recent EM study (Niimi *et al.*, 2008).

Sources of early definitive erythrocytes

The source of yolk sac erythropoietic cells is not clearly resolved. As mentioned above, dorsal aorta-derived cells have been suggested to be able to take up the yolk sac niche and undergo erythropoiesis (Beaupain *et al.*, 1979; Dieterlen-Lievre, 1975; Lassila *et al.*, 1978; Lassila *et al.*, 1982; Martin *et al.*, 1978). This in principle may happen, but currently with no strong evidence either for or against it. Judging from the relative timing and scales of hematopoietic cell generation in the yolk sac and dorsal aorta, it seems reasonable to assume that yolk sac erythropoietic cells during early definitive erythropoiesis are primarily yolk sac-derived. It is another matter, however, to pinpoint which yolk sac cell lineage(s) is involved in early definitive erythropoiesis. Circulating hematopoietic stem cells in E2 and E3, generated from the primitive wave, could be one possible source, although histological and EM studies argue against such a scenario (Bruns and Ingram, 1973; Dantschakoff, 1908; Dawson, 1936; Edmonds, 1966; Lemez, 1964; Lucas and Jamroz, 1961; Romanoff, 1960; Small and Davies, 1972; Sugiyama, 1926). Cells with hematopoietic stem cell potential, residing outside the vasculature after the initiation of circulation at E2, have also been suggested as a possible source for definitive erythrocytes (Dardick and Setterfield, 1978). Our analyses on mitotic activities and timing of the appearance of definitive hematopoietic and erythropoietic clusters in the yolk sac, however, argue against such a stem cell-based hypothesis, at least during the early stages of definitive erythropoiesis.

This leaves two types of yolk sac-derived cells as possible main sources, the vascular endothelial cells and primitive erythrocytes (Fig. 3H). Dantschakoff suggested the venous endothelium as a likely source for definitive erythropoietic cells (Dantschakoff, 1908; Dantschakoff, 1909). This hypothesis is supported by our molecular marker study (Nagai and Sheng, 2008) (Fig. 3 A-C,G). From E2 to E5, yolk sac vasculature undergoes at least two phases of remodeling. Hemodynamic changes initiated by heart pulsation remodels early vascular plexus into arterial and venous trees between E2 and E3, followed by the remodeling from two-dimensional apposing arterial/venous trees to a three-dimensional paired arterial/venous network between E3 and E5 (Eichmann *et al.*, 2005; Gonzalez-Crussi, 1971; le Noble *et al.*, 2004; Nagai and Sheng, 2008; Van Mierop and Bertuch, 1967) (Fig. 4). This second remodeling phase happens in the context of the sinking of major arterial vessels into the yolk, together with associated yolk sac endoderm (Fig. 3G). Smaller venous vessels fuse and come to lie above sunken arteries, and form main paired venous vessel for each artery. Yolk sac definitive erythropoiesis is initiated during this second phase of yolk sac vascular remodeling (Nagai and Sheng, 2008). Hematopoietic foci are observed in venous vessels adjacent to large arteries at E3.5-4, with globin positive definitive erythropoietic cells appearing at E4-4.5 and entering the circulation at E4.5-5. Early definitive erythropoiesis in the yolk sac takes place both intra-vascularly and extra-vascularly in association with remodeling venous vessels adjacent to major arteries and in association with smaller vessels (Fig. 3 B,C). These observations suggest that yolk sac endothelial cells can give rise to erythropoietic cells, either by dedifferentiation first into a hemangioblast type cells or by direct transdifferentiation (Fig. 3H). Such a hypothesis, that hematopoietic cells can be generated from morphologically endothelial shaped cells, is in a sense in agreement with the recent finding of direct generation of hematopoietic cells from the endothelium of dorsal aorta (Bertrand *et al.*, 2010; Boisset *et al.*, 2010; Kissa and Herbomel, 2010). Dantschakoff went one step further and commented that all early vascular endothelial cells possess hemato-

poietic potency, which is exhibited or restricted differentially in different locations (Dantschakoff, 1909).

The other possibility, that primitive erythrocytes may dedifferentiate into definitive erythrocytes, was also mentioned by Dantschakoff (Dantschakoff, 1908) and others (Lemez, 1964; Romanoff, 1960; Sugiyama, 1926) (Fig. 3H). Our analyses indicate that by the end of E3, circulating primitive erythrocytes still retain prominent hematopoietic marker expression (Nagai and Sheng, 2008). These cells are in the process of primitive erythrocyte maturation as discussed earlier, and exhibit primitive erythrocyte hemoglobin profile. During the period of E4, hematopoietic markers are rapidly downregulated in circulating primitive erythrocytes, and definitive hematopoietic/erythropoietic clusters appear in the yolk sac venous vessels and small capillary vessels (Nagai and Sheng, 2008). Interestingly, it is also during this period that most primitive erythrocytes have been reported to undergo the last mitotic division among on average six divisions from the earliest megaloblast/hemocytobalst stage, and enter post-mitotic stage (Campbell *et al.*, 1971; Hagopian and Ingram, 1971; Weintraub *et al.*, 1971) (Fig. 4). It may therefore be interesting to test in future experiments whether at least part of definitive clusters is formed by the dedifferentiation of primitive erythrocytes which do not enter the post-mitotic phase (Fig. 3H). Although currently without direct evidence in the context of chicken yolk sac definitive erythropoiesis, similar hypotheses with regard to differentiation plasticity and potentials have been proposed in other systems (Dzierzak, 2002; Graf, 2002; Kingsley *et al.*, 2006; McNagny and Graf, 2003; Orkin and Zon, 2002; Prindull and Fibach, 2007).

Late definitive erythropoiesis

The picture becomes more complex as embryonic development proceeds. Chimera studies suggest that contribution from intraembryonically-derived definitive erythrocytes, at least by origin, gradually increases, and by E12-13 surpasses the contribution from extraembryonically-derived cells. The major embryonic erythropoietic niche, the bone marrow, has just started active generation of definitive erythrocytes by this time (Dantschakoff, 1909; Metcalf and Moore, 1971). It is possible that some intraebmryonically-derived hematopoietic cells find their erythropoietic niche during mid-incubation period in the yolk sac (Beaupain *et al.*, 1979; Dieterlen-Lievre, 1975; Lassila *et al.*, 1978; Lassila *et al.*, 1982; Martin *et al.*, 1978). Some reports suggested that the yolk sac remains as the major niche for definitive erythropoiesis until E20 (Bruns and Ingram, 1973; Dantschakoff, 1908). A recent histological and EM study suggested this process lasts until E19 (Niimi *et al.*, 2008). In our analysis using molecular markers, prominent erythropoiesis in the yolk sac continues at least until E10 (Fig. 3 D-F), which wanes significantly by E15 (Nagai and Sheng, 2008). Combining these data, it is likely that some time between E12 and E15, the bone marrow takes over as the major erythropoietic organ, although the yolk sac continues to contribute to this process until hatching (Fig. 4).

These studies, although showing that the yolk sac remains a major niche even for late definitive erythropoiesis,

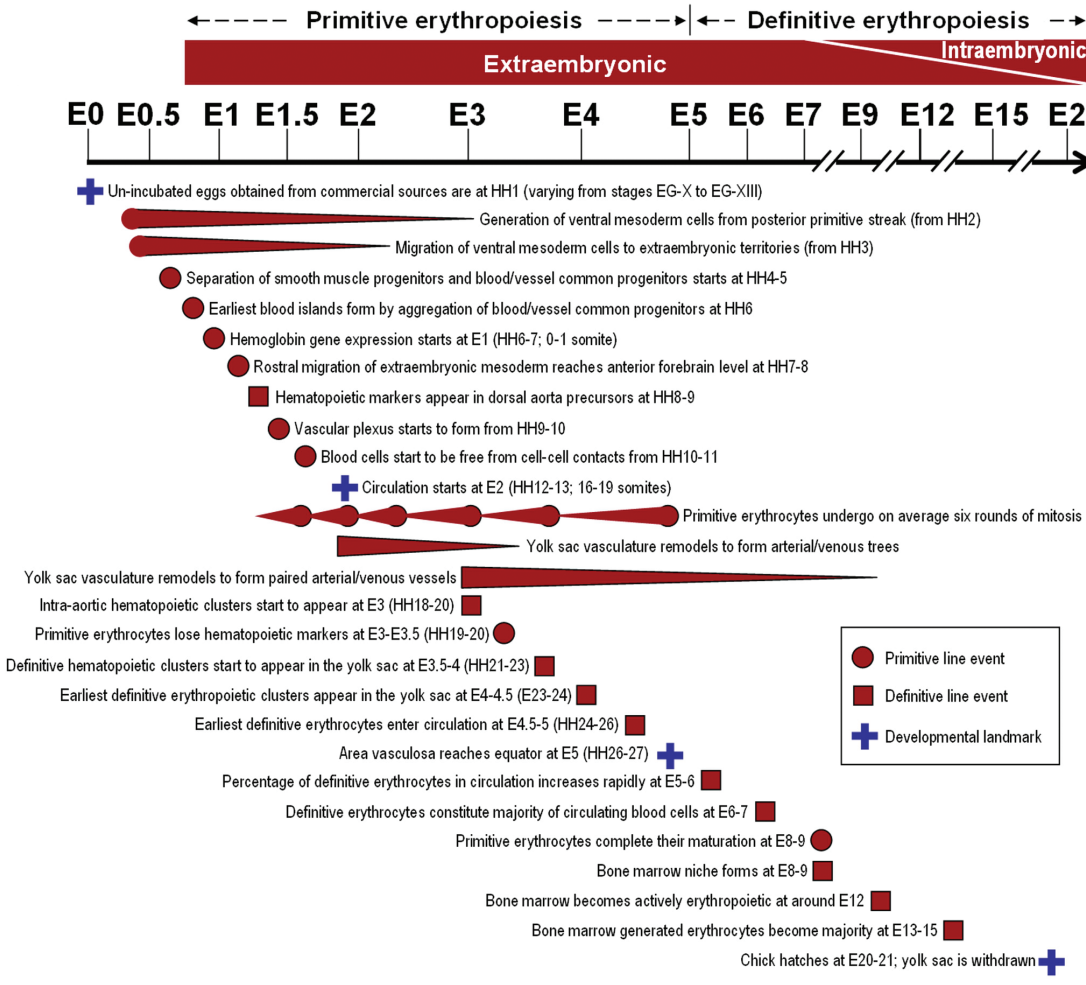

Fig. 4. A timeline of primitive and definitive erythropoiesis in the chick embryo. *Key events of erythropoiesis and some of developmental landmarks are listed, with primitive line-related events marked by circles and definitive line-related events by squares.*

did not address the source of erythropoietic cells at late stages. Similar to the situation in early definitive erythropoiesis, the source of cells for later embryonic and post-hatching erythropoiesis remains contested. Erythropoietic cells in the bone marrow are seeded exogenously. Hematopoietic stem/progenitor cells that do not undergoing erythropoiesis, derived from both the dorsal aorta intraembryonically and the yolk sac extraembryonically, are in circulation at least from late E4 (McIntyre *et al.*, 2008; Nagai and Sheng, 2008). It is unclear whether they remain as dormant stem/progenitor cells in circulation or in a transitory niche before the bone marrow niche starts to form at about E9 and becomes receptive for seeding by circulatory stem cells at about E12 (Bruns and Ingram, 1973; Dantschakoff, 1909; Metcalf and Moore, 1971; Romanoff, 1960).

Other intraembryonic erythropoietic organs, including the liver and spleen, make relatively minor contributions to definitive erythropoiesis during embryonic development. The liver, a major erythropoietic organ in mammalian embryos, is not considered to contribute significantly to definitive erythrocyte generation in chickens. It is noteworthy that development of chicken embryonic liver coincides with the initiation of yolk sac erythropoiesis. Large bilateral yolk sac veins, also known as omphalomesenteric veins or vitelline veins, merge as ductus venosus before reaching the sinus venosus part of the heart. Hepatic vessels are derived from out-branching of ductus venosus endothelium. At E3.5, a very small percentage of extraembryonic blood entering the heart goes through the liver. By E7, however, all yolk sac-derived blood needs to pass through liver tissue as the ductus venosus breaks down after hepatic vessel maturation. Indeed, erythropoietic cells are detected in the liver at E7 both intravascularly and extravascularly (Haff, 1914; Karrer, 1961; Romanoff, 1960; Wong and Cavey, 1993). Erythropoiesis in the liver, however, never becomes very active and stops by the end of E9 (Haff, 1914; Romanoff, 1960). Similarly, the spleen serves as a minor erythropoietic organ for a few days during mid-incubation period (Romanoff, 1960; Yassine *et al.*, 1989). The relatively minor role of liver and spleen as embryonic erythropoietic organs in chickens, however, may not be a general phenomenon in birds, as in passerine birds, such as starling, these two organs were reported to participate in erythropoiesis throughout embryogenesis and for a few weeks after hatching (Romanoff, 1960).

A strong argument for yolk sac origin of hematopoietic cells seeding the bone marrow came from parabiosed and twinned chick embryos of opposite sex (Metcalf and Moore, 1971; Moore and Owen, 1965; Moore and Owen, 1967), in which approximately one fourth to a half of hematopoietic cells in the bone marrow at late embryonic stages are reciprocally derived from opposite sexes. This hypothesis was supported by experiments in which yolk sac-derived cells were injected into irradiated chick embryos, showing that yolk sac cells are capable of contributing to post-hatching bone marrow hematopoietic cell population (Lassila *et al.*, 1978; Metcalf and Moore, 1971; Moore and Owen, 1967). Interpretation of these experiments, however, has to take into consideration the techniques used. For instance, scoring of mitosis at metaphase is not necessarily indicative of hematopoietic cell fate. More importantly, the timing of parabiosis (ranging from E4 to E8) would not distinguish yolk sac-generated from intraembryonically-generated hematopoietic cells in circulation. The age of yolk sac donor (E7) for irradiated embryos may include intraembryonically-generated hematopoietic cells either in circulation or temporarily taking up the yolk sac niche. Studies using chick/quail (Beaupain *et al.*, 1979; Dieterlen-Lievre, 1975; Dieterlen-Lievre, 2005; Martin *et al.*, 1978) and chick/chick (Lassila *et al.*, 1978; Lassila *et al.*, 1982) chimeras presented an opposite picture. Quail or chick embryos ranging from HH9 to HH16 were grafted to chick yolk sacs, and relative contributions of graft and host cells to circulating blood or bone marrow were analyzed. Increasing percentage of donor (embryonic) cells was detected in circulation as chimeras developed and after E17 made up the majority of cells in the bone marrow. These experiments overcame some of the problems in parabiotic/twin analyses, but were also limited by the relatively small sample number, reliability in identifying donor/host cells as hematopoietic cells and variations in the age and graft size of donor embryos, all of which could influence the interpretation. Nevertheless, the latter set of observations suggested that intraembryonically-derived hematopoietic cells make an increasingly significant, and possibly exclusive, contribution to the late embryonic and post-hatching definitive erythrocyte population. It is still unclear, however, whether hematopoietic cells detected in the dorsal aorta during early embryogenesis represent the hematopoietic stem cells seeded in the bone marrow.

An additional possible source of definitive hematopoietic stem cells, which so far has not received careful investigation, is the blood islands around regressed primitive streak near the tail bud. As discussed earlier, the distinction between embryonic and extraembryonic tissues is not clear-cut, especially in the posterior part of the developing embryo. New blood islands are continuously being formed in this area even at E3 when circulation has been well established. Whether to consider these blood islands as embryonic or extraembryonic may be a semantic issue. Molecularly, however, these blood islands can be distinguished from those generated earlier and located more peripherally (unpublished data) (Minko *et al.*, 2003). It will be interesting to test whether these hematopoietic cells may have different differentiation potentials from more conventionally-defined and yolk sac-located hematopoietic cells.

Conclusion

The chick embryo has been and remains to be a superb model system for studying developmental hematopoiesis. It has played important historical roles in providing descriptions and conceptual frameworks fundamental to the field. With diverse model systems and rich resources of molecular and genomic information, elucidation of many currently unresolved questions concerning vertebrate hematopoietic development can be anticipated in the near future. The chick model can continue to make important contributions to this endeavor.

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