Issues in Development

Commentary

The Role of Cell Migration in the Ontogeny of the Lymphoid System

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ABSTRACT

The foundations of experimental hematology were laid by histologists, and while their contributions were enormous, they were limited in their interpretation of very dynamic processes by the static nature of the methodology. The middle of the twentieth century saw the introduction of techniques for hematopoietic cell marking and development of *in vitro* and *in vivo* assays for primitive hematopoietic cells, allowing dynamic studies of hematopoiesis. Paralleling this was an understanding of cellular immunology with the discovery of the role of the thymus and the identification of T and B lymphocyte lineages. In the 1960s a series of ontogenetic studies in birds and subsequently in mice revealed that hematopoietic and lymphoid development involved migration streams of primitive cells that colonized developing primary lymphoid organs as well as spleen, marrow, and liver. The yolk sac was proposed as the ultimate origin of these lympho-hematopoietic precursors. Subsequent studies identified a region associated with the dorsal aorta as the primary site of “definitive” stem cells. These opposing views are currently achieving a compromise that recognizes that both sites contribute stem cells involved in seeding the developing tissues. The clear distinction between the local origin of the inducing microenvironment provided by the endoderm or by stroma derived from mesenchymal stem cells of mesodermal origin, and the immigrant origin of the hematopoietic stem cells and progenitors, raises intriguing questions in the current climate of stem cell plasticity, cell fusion, and discovery of stem cells in adult marrow with the capacity to generate hematopoiesis as well as other mesodermal, ectodermal, and endodermal lineages.

INTRODUCTION

THIS COMMENTARY is in part a personal historical perspective combined with a current review of the field, and in part a rebuttal to a commentary by Dr. Anastassova-Kristeva that appeared in this Journal (1) in which it was proposed that the currently accepted dogma of a common “lymphohematopoietic” stem cell origin of

the lymphoid and hematopoietic system was in error. That commentary cited a publication of mine as supporting this position, to quote: “Moore and Owen (2) found that the thymocytes are produced from stem cells intrinsic to the thymic anlage by direct transformation of endodermal epithelial cells.” This was a misquotation that completely reversed my conclusions. I was particularly pleased with this paper that was published in 1967 be-

cause it was the culmination of my thesis work and basically challenged the then current dogma that thymic lymphoid cells arose by direct transformation of epithelium (3,4). What I believe we clearly showed in that paper was that thymocytes are produced from stem/progenitor cells extrinsic to the thymic anlage that had exited from the circulation and invaded the thymic epithelial anlage. The epithelium did not form lymphocytes but provided the stromal environment supportive of thymic lymphopoiesis. We also showed in the avian bursa of Fabricius, where B lymphocytes first develop, that the process also involved colonization of the epithelial buds by blood-borne stem/progenitor populations (5), rather than by intrinsic epithelial transformation as proposed by then current dogma (6). A later study of thymic development in *Xenopus* was initially interpreted as supporting the intrinsic epithelial origin of thymocytes (7,8); however, subsequent studies by members of this group clearly showed an immigrant origin for thymic lymphoid precursors (9,10). Other protagonists of the epithelial transformation theory also reversed their position in favor of an immigrant stem cell (11–13). The nature and origin of the cells that colonized the primary lymphoid organs has been a subject of debate and intense investigation over the last forty years, but there has been no serious challenge to the concept of circulating stem/progenitor cell populations migrating into organ anlagen and initiating hematopoiesis in fetal liver and bone marrow and lymphopoiesis in thymus, bursa of Fabricius, and spleen (2,5,14–17).

A simple question still remains unanswered: namely, why do stem and progenitor cells migrate through the circulation, and why are all lymphohematopoietic tissues dependent upon such extrinsic cells? A similar question can be posed in the case of primordial germ cells that also migrate through the embryo to the developing gonads. The concept of specific developmental niches or microenvironments can be evoked. These provide extrinsic signals that determine pluripotential stem cell fate. The yolk sac blood islands, generating both primitive and definitive generation stem and progenitor cells but supporting only primitive erythropoiesis, is one niche. The floor of the aorta is another niche where adult-type stem cells arise as well as definitive lymphoid and myeloid progenitors. The fetal liver provides the environment for extensive hematopoietic and B cells differentiation as well as stem cell amplification, while the epithelial niche of the thymus supports T lymphoid expansion and specialization but not stem cell survival. In this concept the lymphomyeloid stem cell is a *tabula rasa* whose differentiation fate is determined by the environment into which it has migrated. It is likely that this is not valid for the cells colonizing primary lymphoid organs since there is growing evidence for some degree of lymphoid commitment occurring prior to colonization, and consequently

some degree of selection of colonizing cell types (e.g., by selective chemoattraction). An extraordinary array of extrinsic signals are provided by these microenvironments including members of the Wnt and hedgehog family, Notch ligands and a spectrum of hematopoietic growth factors, cytokines and chemokines, cytoadhesion molecules, and extracellular membrane components. A combination of functional and genomic analysis is beginning to unravel the nature of specific microenvironments and explain their ability to orchestrate the complex patterns of proliferation, self-renewal, and multi-lineage specification that characterize lymphohematopoiesis.

HISTORICAL PERSPECTIVE

Early attempts to understand the complexity of hematopoietic and lymphoid differentiation were limited by the available tools—the microscope and the stained slides revealed the static morphology of the tissues. Unfortunately this did not prevent the early observers from making dynamic interpretations of cell lineages and origin. In general they proved reasonably accurate when dealing with the later stages of hematopoietic differentiation. Problems arose when morphologically undifferentiated blast cells or mononuclear cells were endowed with various potentialities. There was also a great deal of controversy concerning “transitional” stages between, e.g., mesenchyme and endothelium and hematopoietic lineage, and between epithelium and lymphocyte. This was followed by dynamic studies that permitted analysis of cell fate in transplantation experiments designed to study radioprotection or immune tolerance.

In 1949 Jacobson et al. (18) made a seminal observation that shielding of the spleen rendered nonlethal a dose of radiation, which administered to the entire body would have been lethal. A subsequent study showed that injection of bone marrow or spleen cells from a nonirradiated donor had the same effect as shielding the spleen (19). While in hindsight the explanation for these results seem obvious, for some time there was debate as to whether this protection was due to some trophic effect or was due to engraftment of cells. It was only in later studies employing chromosome markers that the cellular basis of this radioprotection was firmly established and mice injected with suspensions of hematopoietic cells were shown to recover because their hematopoietic system, including lymph node, thymus, marrow, and spleen were repopulated by adult bone marrow cells and also fetal liver cells (20,21).

The question was then raised as to whether such trafficking and colonization would occur in nonirradiated animals. Harris and Ford (22) had pioneered a system based upon identifiable cytogenetic differences using the T6 chromosome marker system in the mouse and showed by

thymic grafting and regrafting experiments that there is a movement of cells from the bloodstream through the thymic graft to the lymph nodes. In adult mouse parabiosis studies, again using the T6 marker, a reciprocal exchange of cells between partners was demonstrated in lymph node, spleen, thymus, and bone marrow, and chimerism persisted after the animals were separated (23). It was significant that while spleen and lymph node chimerism was 40–50%, marrow chimerism was only 5% at 4–5 weeks and thymus and marrow chimerism only reached a 15–18% even after 30 weeks of union, suggesting only a low level of immigrant stem cell influx in the adult steady state in these tissues. This important observation was confirmed and extended by studies some 37–39 years later (24,25) indicating that stem cell homeostasis within the adult marrow is primarily maintained by the retention of stem cells derived from the replication events within the marrow, not the homing and engraftment of stem cells from the circulation. The question remained as to whether vascular interchange of cells between hematopoietic and lymphoid organs occurred during development.

A crucial observation had been made by R.D. Owen in 1945 (26) in his discovery that persistent erythrocytic chimerism occurred among dizygotic twin cattle where placental vascular anastomoses had formed early in their development. This was a seminal observation for our future understanding of tolerance. Moreover, it held profound implications with respect to hematopoiesis. Owen's observations prompted me to conclude that hematopoietic cells readily exchanged via the vasculature during development. Among the hematopoietic cells exchanged, I reasoned, there existed a population that stably generated progeny of all lineages—hematopoietic stem cells. If this interchange was not a phenomenon limited to dizygotic cattle, could it also provide a physiological explanation for lymphoid engraftment reported in radiation chimeras? Against this background, I undertook a series of experiments to investigate the validity of the hypothesis I was developing. Hasek (27) had devised a technique for bringing about a vascular fusion between the chorio-allantoic membranes of two chick embryos that led to mutual tolerance in the chicks after hatching, providing me with an experimental system to evaluate migration streams. By varying the time of onset of establishment of the vascular anastomosis and the time of evaluation of the developing embryos hematopoietic and lymphoid tissue I believed that I could determine with some accuracy the timing of cell migration via the circulation and the role of cell migration, if any, in establishing lymphopoiesis or hematopoiesis from the outset. However, I needed a stable marker for tracking the interchange of cells between the parabiosed embryos. This was provided by John Owen, my thesis advisor, who devised an improved method for preparing avian metaphase

chromosome spreads that allowed accurate discrimination of male from female (28). In a series of papers between 1965 and 1967 Dr. Owen and I demonstrated that extensive, durable chimerism resulted if vascular anastomosis was established prior to the onset of hematopoiesis in marrow or spleen, or prior to onset of lymphopoiesis in thymus and bursa of Fabricius (2,5,14–17,26). Significantly, thymic chimerism was not observed if parabiosis was delayed till after the onset of thymic lymphopoiesis, suggesting timed waves of colonization. As initially formulated we proposed that yolk sac blood islands were the primary source of these migratory cells based upon the fact that this was the first site of hematopoiesis and remained a major site of hematopoiesis at the time the primary lymphoid organs were undergoing colonization (2,4,6–9).

Sir MacFarlane Burnet, who had received the Nobel Prize with Peter Medawar for his clonal selection theory of immune development, invited me to be his postdoctoral fellow at the Walter and Eliza Hall Institute of Medicine in Melbourne, which, at the time was the mecca of immunology research and retains a formidable position in immunology and hematology research to this day. After two years, I decided the challenge before me lay not in basic immunology but in the broader field of experimental hematology. At that time the *in vitro* colony forming cell (CFC) assay that had been developed by Ray Bradley and Don Metcalf in Melbourne (29) provided a quantitative way of measuring an early hematopoietic progenitor. The relationship of the CFCs to the spleen colony forming unit (CFU) discovered by Till and McCulloch in 1961 (30) was, however, unclear. We used the continuous density gradient cell separation system developed by Ken Shortman at the Hall Institute to physically separate CFUs from *in vitro* CFC (31–33) and proposed a parent-progeny relationship, with the CFUs representing hematopoietic stem cells and the CFC being committed progenitors derived from them. This concept held for many years, until it was determined that the CFUs (at least the great majority of such cells) were not long-term repopulating stem cells (34).

We applied these two assays to determine when in development either cell type originated (17,35). We showed that CFC that had the cytokine responsiveness and differentiation potential of definitive CFU-GM, preceded CFUs in YS but that at later stages of development CFUs and long-term repopulating stem cells appeared in YS, followed by fetal liver, then spleen, and finally marrow. In organ culture of embryo and yolk sac separated at E7.5 we observed extensive erythropoiesis with generation of definitive CFU-GM in the yolk sac itself at levels equivalent to cultures with YS attached to embryo, whereas in embryo alone there was no evidence of hematopoiesis, nor detectable CFC formation in the absence of the yolk sac. We believed that this was evidence that definitive

hematopoiesis originated in the yolk sac and continued after YS hematopoietic stem/progenitor cells migrated into the embryo. We then showed in a series of grafting and transplantation studies that murine fetal liver was colonized by immigrant cells at E10 at the ~28 somite stage of development (36). Subsequent studies by Dieterlen-Lievre and associates (37–40) exploited differences in nuclear morphology of the chick and quail embryos to demonstrate, in hybrids, an intraembryonic origin of lymphoid precursors in the para-aortic region of early embryos. Medvisky et al. (41–43) first provided convincing evidence for the origin of primitive hematopoietic stem cells capable of fully reconstituting the lymphomyeloid system when transplanted into adult mice. These cells arose in the floor of the dorsal aorta in the aorta-gonad-mesonephros (AGM) region of the E10.5 mouse embryo.

The intense debate over the virtues of the yolk sac versus the intraembryonic site as sources of cells that subsequently colonized the later developing lymphoid and hematopoietic tissue has continued to the present day and obscures the significance of the embryonic stem cell migration streams that we defined years ago. The following sections review the current state of knowledge of hematopoietic, lymphoid, and endothelial ontogeny. Since I present my review with a personal bias, I direct interested readers to other reviews in this area that have been published in this Journal in recent years (44–47).

DEVELOPMENT OF THE YOLK SAC

In the mouse, mesodermal cells destined for extraembryonic sites exit the posterior primitive streak and subdivide the embryo into three separate cavities by the neural plate stage (E7.5). The central cavity, the exocoelom, becomes completely lined with mesoderm and visceral yolk sac (YS) forms where this is adjacent to visceral endoderm. The posterior primitive streak-derived mesoderm receives signals from the visceral endoderm that involve Indian hedgehog, and smoothened (Smo), a receptor component essential for signaling, acting via induction of bone morphogenic protein-4 (BMP-4) in mesoderm, that in turn induces hematopoietic and vascular specification and blood island development (44, 48–50). Vascular endothelial growth factor A (VEGFA) plays a pivotal role in the first step of endothelial and hematopoietic development in the YS and at the onset of gastrulation is expressed in the YS visceral endoderm and mesothelium (51). Its receptors, Flk-1 and Flt-1, are expressed on the hemangioblasts and angioblast and Flt-1 is expressed on the HSC developing in the blood island (46,52,53).

The YS is the site of primitive generation erythropoiesis and macrophage production. The wave of primitive eryth-

ropoiesis begins in the mouse YS at E7.5 with nucleated red cells producing embryonic globin predominating in the circulation through E14.5 with eventual replacement by fetal liver-derived red blood cells (RBC) expressing adult globins by E15.5–16.6. The YS is also the first site of definitive multilineage, and myeloid and erythroid-lineage restricted, progenitor cell production (35,54,55). Definitive erythroid (BFU-E) and myeloid (CFU-GM) progenitors and mast cell precursors are detected in the YS at E8 (35,55). They appear not to differentiate locally but most likely migrate to the fetal liver where BFU-E/CFU-E initiate definitive erythropoiesis (56). A population of high proliferative potential (HPP) CFC were detected at E8 (1–8 somites), exclusively in the YS, and this remained the main site of expansion (>100-fold) of these multipotent precursors until E10.5–11.5 when there was a dramatic increase of HPP-CFC in the circulation and liver, with a concomitant drop in the YS (55). Upon secondary replating these HPP-CFC exhibited the stem cell features of self-renewal and potential to generate definitive erythroid and macrophage progeny (45,46). Separation of YS and embryo at an early E8 stage, before a common circulation is established, showed that HPP-CFC were found exclusively in the YS, and examination of the blood at E8.5 suggested that they entered the circulation from YS together with the first erythroblasts.

The YS does not contain cells capable of long-term multilineage engraftment of adult irradiated mice until after such cells have appeared in the AGM region at E10.5, but they appear by E11 in the YS and are present till E13 (35,43,57). It has been argued that the AGM region is the sole site of origin of “definitive” HSC capable of engrafting adult recipients, and the presence of these cells in the YS at later stages represents an immigrant population (58–61). However, total body quantitation and tissue distribution analysis of competitive repopulating HSC detected by limiting dilution analysis argues for two independent sources of “definitive” HSC, with both AGM and YS participating in seeding the fetal liver (43). Support for this view was obtained by Matsuoka et al. (62) who generated both CFUs, and HSC that engrafted adult mice, following co-culture of either E8–8.5 YS or intraembryonic splanchnopleuric mesoderm (P-Sp) on a stromal cell line generated from the AGM region.

HSC are defined as cells with multilineage (lymphomyeloid) differentiation potential and extensive self-renewal potential. By this criteria, the YS contains CD34⁺, c-Kit⁺, CD38⁺ HSC as early as E9 that when injected directly into the liver of busulphan-conditioned neonatal mice, gave long-term lympho-myeloid reconstitution (54,55,63). Secondary engraftment in sublethally irradiated mice demonstrated that the YS-derived HSC acquired normal marrow homing properties. The first detection, in the AGM region at E10.5, of HSC capable of repopulating adult mice (41–43) does not *a priori* exclude

YS HSC in playing a significant role in subsequent hematopoietic development since the only requirement of the initial HSC population is that it be capable of colonizing the liver, which YS-derived HSC clearly can do (54,55,63). Co-culture of YS on AGM stroma has been reported to confer adult engraftment potential (62), suggesting an inducible function for stromal cells in this region, particularly mesenchymal cells on the floor of the dorsal aorta. As currently envisaged, this inductive influence confers HSC potential in locally generated hematopoietic clusters on the floor of the dorsal aorta (*vide infra*), but it is conceivable that early HSC exiting the YS may traffic into the dorsal aorta and come under the influence of the AGM stroma, and acquires adult engraftment potential. The molecular basis for acquisition of adult engraftment potential may involve upregulation of HOX genes since transduction of HOXB4 into cells isolated from precirculation YS (E8.25) endowed them with long-term lymphomyeloid engraftment capacity in adult irradiated mice (64).

LYMPHOID POTENTIAL OF YS CELLS

There has been a long, and as yet unresolved debate concerning the role of the YS in generating B cell progenitors. Early studies with Ig allotype markers showed that YS cells transplanted into adult mice generated B cells (65), and in utero injection of E8-10 YS resulted in post-natal B and T chimerism (66). Chromosome marked E11 YS cells were shown to provide long-term lymphoid (T and B) and myeloid engraftment in mice (35). In a series of studies, Auerbach et al. (12,13,67,68) showed that AA4.1⁺ cells from YS as early as E8-E9, when co-cultured on marrow stroma or YS endothelial lines could generate cells with B and T potential as measured in vitro and when transferred in vivo into immunodeficient mice.

Cumano et al. (69) initially reported that AA4⁺, Sca-1⁻ cells in both yolk sac and embryo at E8.5 were able to generate both T and B cells in an S17 stromal co-culture with IL-7 and c-Kit ligand. In a subsequent study Cumano et al. (70) separately cultured mouse embryo and YS at the presomite, pre-vasculature E7.5-8 stage for 48 hrs and observed B cell differentiation only in the embryo. B lymphoid potential was observed in cultured YS obtained after the circulation was established, favoring their view that the YS is colonized by P-Sp-derived lymphoid progenitors. The absence of definitive hematopoietic progenitors in their cultured YS, yet their presence in the YS *in situ*, suggests that the culture conditions were deficient for maintenance of a normal pattern of lymphohematopoiesis, rendering a negative result inconclusive. Ogawa et al. (71) reported B cell progenitors developing in cultures of E9.5 embryos but not of YS, although excessive production of mast cells in the latter

cultures may have inhibited lymphoid differentiation. Nishikawa et al. (72) isolated VE-cadherin⁺, CD45^{-ve} "endothelial" cells from E8.5-9.5 embryo and YS and showed B cell differentiation from both sources on OP9 stroma in the presence of IL-7, an important early B cell growth factor.

These variable and often conflicting data can be attributed, in part, to culture variables such as whole tissue cultures versus dispersed cells, the use of different stromal lines, and particularly the use of supplemental cytokines such as IL-7 and c-Kit ligand. Certain of these conditions may provide an environment capable of supporting lymphoid differentiation from "hemogenic" endothelium or hemangioblasts; others may support differentiation from lymphomyeloid stem cells and pMix; while others can expand only those cells that have undergone B cell commitment (pB or pB/macrophage). By restricting the criteria for a committed B cell progenitor to cells expressing CD45, and probably CD19, IL7R α and c-Kit, these cells first appear within the E10 AGM region where they may traffic to the YS as well as the early liver. It is probable that more primitive cell populations in the YS can migrate into the embryo and undergo pB commitment in the AGM region or early liver.

DEVELOPMENT OF HEMATOPOIESIS IN THE AORTA-GONAD MESONEPHROS REGION

In the human embryo, hematopoietic clusters of CD34⁺ cells appear in the aorta at 27 days gestation, 8-9 days after initiation of YS hematopoietic, and 6 days after the onset of the circulation (Fig. 1) (58,59,73). Ultrastructural studies show that the endothelial basal lamina is intact where it interacts with the cells in the hematopoietic clusters, and the hematopoietic and endothelial cells are interconnected by tight junctions (74,75). The precursors of the hematopoietic clusters in the periaortic region were considered "endothelial" based on expression of CD34 and CD31 (PECAM-1), *Ulex Europaeus* lectin binding, uptake of acetylated low-density lipoprotein (Dil-Ac-LDL), and absence of CD45 (45-47,71). This blood-forming "endothelium" is present in the human embryo AGM region at 28 days with a peak frequency at 31 days and absence by 44 days (76). Culture of the para-aortic splanchnopleura (P-Sp) from 21-26 day human embryos on marrow stromal cells generated hematopoietic cells, demonstrating the hematogenic potential of P-Sp mesoderm that precedes the appearance of "hemogenic endothelium" (59). The pre "endothelial" mesodermal precursor is CD34^{-ve}, CD45^{-ve} (20), and VEGFR-2/KDR⁺ (77).

In mice, hematopoietic clusters appear at E10.5 in the ventral floor of the dorsal aorta. They appear to be in direct contact with underlying mesenchyme where the en-

FIG. 1. Photograph of a 5-week-old human embryo. Ao, dorsal aorta; AL, anterior limb rudiment; L, liver; H, heart; YS, yolk sac. Enlarged view of the dorsal aorta from the same embryo immunostained for CD34 expression, showing CD34⁺ endothelium and the ventral thickening of the vessel wall resulting from accumulation of rounded CD34⁺ hematopoietic cells that are also CD45⁺. Nucleated erythrocytes are present in the lumen. Photo courtesy of Dr. Bruno Péault. From Tavian M, L Coulombel, D Luton, HS Clemente, F Dieterlen-Lievre, and B Péault. (1996). Aorta-associated CD34⁺ hematopoietic cells in the early human embryo. *Blood* 87:67–72. Copyright© American Society of Hematology, reproduced with permission (73).

endothelium is interrupted. VE-cadherin-expressing cells in the AGM have hematopoietic potential, and VE-cadherin is expressed on the luminal aspect of vascular endothelium but not in CD45⁺ aorta-associated hematopoietic clusters (45,72,76,78). A discrete region of densely packed mesenchymal cells lies beneath ventral floor of the dorsal aorta and is 3–4 layers thick in mouse and 5–7 layers in human (58,74,75). These mesenchymal cells are interconnected by tight junctions, express smooth muscle α -actin (SM α -A), and may be precursor of vascular smooth muscle. BMP-4 is polarized to the ventral wall of the dorsal aorta in the mesenchymal layer underlying the intra-aortic hematopoietic clusters where it could induce P-Sp mesoderm to become hemangioblasts and HSC (79). Medivinsky et al. (41) identified the mouse AGM region at E10.5 as a source of CFU-S, preceding their appearance in YS. CFUs precursors were present in both AGM and YS by E9 since cultures of either tissue at this stage generated CFU-S (42). In vivo engraftment was obtained with cells from the E10.5 dorsal aorta and vitelline/umbilical artery (80,81). Quantitation of long-term repopulating stem cell numbers by limiting dilution assay has shown that they first appear in the AGM at E10.5 and are present in equal numbers in AGM, YS, circulation, and early liver rudiment by E11 (43). HSC capable of long-term lymphomyeloid engraftment could be generated in organ culture of P-Sp isolated prior to the onset of the circulation (E7.5–8), whereas cultured YS failed to generate such HSC (61). However, Matsuoka et al. (62) were able to generate repopulating HSC in co-culture of equally early P-Sp and YS with a stromal line (AGM-S3)

derived from the E10.5 AGM region. This line may have some unique properties since in a study of 100 stromal lines generated from AGM subregions, most were supportive of adult HSC expansion but none were able to induce development of engraftment HSC when co-cultured with CD34⁺ cKit⁺ cells from E10 AGM or YS (82).

The various inductive influence of the subaortic mesenchyme could cause bipotential precursors located in the floor of the aorta to preferentially adopt a hematopoietic fate. Alternatively, endothelial cells may de-differentiate and switch to a hematopoietic fate in response to local or transient signals. A third possibility is that hemangioblasts or their precursors may migrate secondarily into the aortic wall, either from the underlying mesenchyme or from the circulation, in response to a chemokine gradient. Endothelial cells, angioblasts, and HSC express CXCR4, and mesenchyme adjacent to the dorsal aorta expresses high levels of its ligand SDF-1, indicating a role for this chemokine pathway in both vasculogenesis and migration of hemangioblastic precursors to the floor of the aorta (83).

The *Runx1/AML1* gene plays a critical role in establishment of definitive hematopoiesis in the AGM. *Runx1* expression is initiated in mesenchymal cells at the distal tip of the allantois, and endothelial cells in ventral portion of paired dorsal aorta at E8.5 and in both endothelium and mesenchyme of the ventral AGM and in the intra-aortic hematopoietic clusters between E9.5–11.5 (84). In *Runx1/LacZ* mice, some 30% of the cells recovered from the AGM and the vitelline and umbilical arteries at E11.5 were *Runx1/LacZ*⁺, and this population contained

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all HSC capable of engrafting irradiated recipients (84). These HSC were initially CD45^{-ve} at E10.5, but by E11.5, HSC activity was present in both CD45⁺ and CD45^{-ve} fractions. The vast majority of HSC was CD41⁺ (85) and were found in both the positive and negative fraction of AGM stained with CD31, Flk-1, or VE-cadherin (84). The expression of Flk-1 on these HSC populations is controversial, with some studies showing expression of the receptor in the hematopoietic clusters (86), others showing that it is barely detectable in hematopoietic clusters but is expressed on hemangioblastic precursors (77), or providing data suggesting that some HSC that lack Flk-1 did engraft indicating downregulation of Flk-1 on CD45⁺ intra-aortic clusters (84).

In *Runx1*-deficient mice, HSC and intra-aortic clusters were absent, while in *Runx1* hemizygous mice, hematopoietic clusters were reduced in number and size and HSC numbers and distribution were altered, indicating a gene dosage effect (84). Haploinsufficiency of *Runx1* results in an earlier appearance of engraftable HSC in the YS with E10 YS cells now capable of reconstituting the hematopoietic system of irradiated adult mice (87). At the same time there is premature termination of HSC activity in the AGM explants consistent with a change in the balance of HSC emergence, migration, and/or maintenance. With hemizygous levels of *Runx* the YS may autonomously generate HSC simultaneous to their generation of HSC in the AGM; alternatively AGM may autonomously generate abundant HSC that immediately and rapidly migrate to the YS where they are detected in abundance, or *Runx1* insufficiency blocks emigration of HSC from the YS resulting in accumulation there and a deficiency in the AGM.

In birds at E2 the aortic endothelium consisted entirely of CD45^{-ve}, VEGFR-2⁺ flat endothelial cells. The first intraembryonic hematopoiesis occurs at E3 in endothelium-associated intra-aortic clusters of markedly basophilic cells with prominent nucleoli. CD45⁺ VEGFR-2^{-ve} hematopoietic progenitors are detected in the aortic wall at E3–4 (88). Subsequently (E6–8) hematopoiesis occurs in diffuse hematopoietic “para-aortic foci” located in the dorsal mesentery ventral to the aorta (89,90). Marker studies showed that the intra-aortic clusters developed in situ and not by migration from the YS (91). Acetylated low-density lipoprotein (DiI-Ac-LDL) labeling indicated that the hematopoietic clusters originated from aortic endothelium, while retroviral vector marking indicated that the para-aortic foci were derived from intra-aortic clusters (89,90). Mesodermal subdivision transplantation showed that the endothelium of floor of the dorsal aorta develops from splanchnopleural mesoderm and generates progenitors with dual hematopoietic and angiogenic potential, while endothelium of the aortic roof and sides arise from somite mesoderm that generates “pure” angioblasts (89).

Vascular development is initially asymmetric, centered in YS blood islands, and in the embryo proper where angioblasts begin to coalesce into the aorta. Morphogenesis of blood vessels is marked by a sequential pattern of gene expression in which SCL and Flk1 are expressed first at the angioblast stage, followed by PECAM, CD34, VE-cadherin, and later Tie2, while SCL expression is downregulated in endothelium of mature vessels (92). By the murine 3 somite stage, vascular development has spread through the YS, and extended aortic tubes are visible (93). Beginning at the 4 somite stage, erythroblasts disperse through the distal yolk sac and a few erythroblasts appear in the head and tail region of the embryo where the embryonic and YS vasculature meet. The circulation onset is defined as erythroblast movement within vessels and is initiated at the murine 4–6 somite stage during E8.25.

The respective roles of hemangioblasts as common precursors of endothelium and hematopoiesis versus the plasticity of “hemogenic endothelium” in initiating hematopoiesis in the AGM or YS is debated (45,46,74–76, 79,90,94). This is not simply a semantic issue since the hemangioblast concept recognizes a common undifferentiated precursor, whereas the “hemogenic” endothelium concept recognizes that an endothelial pathway of differentiation has occurred but the cells remain sufficiently plastic to be capable of redifferentiating to hematopoietic lineage. A number of these studies identify endothelium as the source of hematopoiesis based upon isolation of cells expressing markers associated with endothelium (VE-cadherin, CD31/PECAM, Flk-1, Flt-1, Tie-2, *Ulex Europus* agglutinin binding, DiI-Ac-LDL uptake). None of these markers is unique to endothelium and possibly could be expressed on hemangioblasts or pre-hematopoietic mesoderm, while some are expressed on HSC or more differentiated myeloid cells. Direct derivation of hematopoietic cells from mature, already differentiated, endothelium has been reported in Ac-LDL-DiI labeling studies (89,90,95). Following intra-cardiac injection of E10 mouse embryos with Ac-LDL-DiI, staining was found within 1 hr and was confined to CD31⁺, CD34⁺, CD45^{-ve} endothelium along the entire vascular tree (95). At twelve hours after injection, 1.4% of circulating cells were DiI⁺, and of these, 43% expressed the erythroid marker Ter 119 and adult globin, characteristic of definitive definitive erythropoiesis. The rest of the DiI⁺ cells represent committed white blood cells, lineage restricted and multi-potent progenitors as revealed by colony formation, and HSC. The remarkable feature of this observation is the rapidity with which the endothelium differ-

entiate to multipotent HSC, and lineage restricted erythroid progenitors (BFU-e) that in turn differentiate to the extent that they express globin genes, maturation steps that normally require at least 4–6 cell divisions. It is also likely that the transition from hemangioblast to endothelium or HSC is not abrupt, but is a progressive process of increasing restriction. A cautionary note in various marker studies involving endothelial and hematopoietic cells is provided in co-culture experiments with CD34+ cells and tumor necrosis factor- α (TNF α)-treated endothelium, where we observed, by confocal microscopy, a bi-directional transfer of an intact marker protein (Green Fluorescent Protein) between developing dendritic cells and endothelium (96). This interchange was facilitated by the intimate interaction, observed in ultrastructural analysis, between the myeloid dendritic cells which can be partially “internalized” by TNF-stimulated endothelium with the membranes of both cell types remaining intact. This may represent emperipoiesis, a form of transmigration of one cell type through another. A further caveat is that most of the markers that have been considered endothelial-specific can be expressed on hematopoietic cells. Certain monocyte-macrophage-derived cells have proangiogenic effects due to secretion of angiogenic growth factors and express endothelial-associated features (CD31, incorporation of Dil-acLDL, binding of Ulex europus agglutinin), yet still express macrophage features of CD45 and CD14 expression (96). These cells may be comparable to a CD45+, CD11b+, Sca-1+, Tie-2+ partially CD34+ hematopoietic population that is also reported to home to sites of neoangiogenesis and closely interact with vascular endothelial cells (97).

Evidence for the existence of hemangioblasts has come from studies on embryonic stem cells. Culture of embryonic stem cell-derived embryoid bodies with vascular endothelial growth factor (VEGF) and cKit ligand led to development of colonies of blast cells (CFU-BI) which upon secondary replating generated colonies of both primitive generation of erythroid as well as definitive generation erythroid and myeloid lineage, supporting the view that both definitive and primitive generation can arise from a common precursor (98). However, murine ES cells that have *Runx1* homozygously deleted can form hemangioblasts that produce primitive but not definitive hematopoietic cells (99). In addition, blast colonies can be shown to generate endothelium, supporting the view that CFU-BI are hemangioblasts (100). The thrombopoietin (Tpo) receptor c-Mpl can be detected by day 3 of embryoid body formation when hemangioblasts first arise and Tpo alone supported CFU-BI formation and nearly doubled the number of blast colonies generated when combined with VEGF and c-Kit ligand (101). A more primitive embryoid body-derived VEGF-independent type of colony forming cell was subsequently described, the transitional CFC (102) or

pre-blast CFC (103). This type of colony contains cell population undergoing commitment of mesoderm to hematopoietic and endothelial lineages, and cardiomyocytes (102). Individual transitional colonies express a combination of mesoderm, hematopoietic, and endothelial genes (*Brachyury*, *flk-1*, *SCL/tal-1*, *GATA-1*).

Kumano et al. (104) showed hematopoiesis and angiogenesis was severely impaired in E9.5 P-Sp organ cultures established from Notch1-deficient embryos. P-Sp and YS cells from Notch 1+/+ and +/- but not from Notch -/- embryos showed hematopoietic engraftment in busulfan-conditioned newborn mice. Studies with conditional Notch1 deficient BM showed normal hematopoietic engraftment; thus Notch1 acts at the stage of HSC and angioblast generation from hemangioblast in both P-Sp and YS but is not required for expansion or maintenance of HSC (104).

A homeobox gene termed Hex has been shown essential for hemangioblast differentiation, and studies of *Hex*-/- embryonic stem (ES) cells and embryos has shown marked defects in definitive hematopoietic progenitor production in YS, and embryoid bodies showed defective endothelial and definitive hematopoietic development (105).

The zebrafish mutation *kugelig* is associated with severe anemia, reduced expression of *SCL*, and total absence of *AML1/Runx1* in blood cells but with normal numbers of *Flk1* + angioblasts and normal vascular development (106). The mutation is in *Cdx4*, a member of the caudal family of homeobox genes that are thought to regulate *Hox* gene expression. A late primitive streak stage *Sdx4* is confined to mesodermal cells of posterior embryo, the allantois, and the YS wall. Results suggest that if hemangioblasts exist in vivo then they must arise before the onset of *SCL* expression, and that *Cdx4* is necessary for this population to differentiate into an *SCL*+ hematopoietic precursor. Alternatively the blood and vascular lineages may arise independently from posterior mesoderm, with *Cdx4* being required solely for specification of hematopoietic fate. Finding that *Scl* over-expression fails to rescue blood development suggests that the pathway acts to make lateral plate mesoderm competent to respond to genes that specify hematopoietic fate (106). Transduction of *Cdx4* into embryoid body cells induced a marked enhancement of hematopoietic differentiation associated with upregulation of *Hox* genes (*HoxB3*, *HoxB8*, *HoxA9*, and particularly *HoxB4*) that are implicated in HSC or progenitor cell proliferation.

DEVELOPMENT OF THE THYMUS

The thymus develops from the endoderm of the third pharyngeal pouch with subsequent epithelial-mesenchy-

mal interactions and Notch signaling participating in thymic organogenesis (2,107). Between E11–E12 in the mouse, the stratified epithelium undergoes morphological changes, beginning to express high levels of MHC II+ and of Delta1, the Notch ligand that plays a critical role in T cell development (107) (Fig. 2). Comparable development of the thymic rudiment is seen at embryonic day ~35 days in human, 7–8 days in the chick embryo, and day 4 in *Xenopus*. Our studies in parabiotic chick embryos demonstrated very high levels of thymic lymphoid chimerism following establishment of a yolk sac vascular union at d6–7 of incubation but not following a later vascular union (2,14). We proposed that the thymic immigrant cells were derived from the yolk sac (2,16), a view supported by studies in which 7-day yolk sac cells were injected into irradiated chick embryos and colonized the thymus (15,39), and by studies on the thymic homing and differentiation of tritiated thymidine-

labeled yolk sac cells (Fig. 3) (17). However sex mismatched YS and embryo chimeras generated at 33–55 hrs of incubation showed that the thymus was populated by cells of the sex of the embryo and not by YS (16). Studies in chick-quail chimeras also showed that the primary lymphoid organs were colonized by cells that originate in the para-aortic region (39,40). Coltey et al. (108) proposed that the avian thymus is colonized by three waves of immigrant cells, the first wave came from para-aortic foci at d6, with subsequent waves at day 12 and day 18 originating from marrow and spleen. These opposing views can be reconciled if the yolk sac is colonized at a very early stage by cells originating in the para-aortic region, and like the mammalian fetal liver, may then serve as a site for expansion of lymphoid progenitors that secondarily migrate to the thymus. In *Xenopus* embryos, diploid ventral blood islands (VBI), analogous to the yolk sac, and the dorsal stem cell compartment (DSC) analo-

FIG. 2. **A.** E11 mouse embryo thymic rudiment. At this stage the rudiment is composed of pale-staining epithelial cells with a central lumen, a remnant of the pharyngeal pouch. Candidate immigrant cells with deeply staining basophilic cytoplasm and prominent nucleoli (arrowed) are present within the epithelium and adjacent to it. Giemsa stain. $\times 450$. **B.** Basophilic blast cell in 11 day thymic rudiment. $\times 1000$. **C.** E12 mouse thymic rudiment with numerous large basophilic cells within the circumscribed epithelial rudiment at a stage prior to penetration of the epithelium by vasculature. $\times 450$. **D.** Basophilic cells in E12 thymus. Note the deeply stained cytoplasm, prominent nucleoli and Golgi apparatus (arrowed). $\times 1000$.

FIG. 3. Tritiated thymidine-labeled avian embryonic yolk sac (E7), embryonic blood (E13), embryonic spleen (E14), or embryonic marrow (E18) cells were injected intravenously into 8-14 day chick embryos. All these sources showed homing of labeled cells to the E8-9 thymus and E14 bursa of Fabricius. Comparably labeled embryonic thymus or bursal cells did not home to these sites. **A.** Autoradiograph of a section of the E9 thymus 24 hrs after injection showing a pair of labeled cells (arrowed) (probably daughter cells of a cell division) in the perithymic mesenchyme adjacent to the epithelium. H&E stain $\times 1000$. **B.** Autoradiograph of a section of the E9 thymus 24 hrs after injection showing heavily labeled basophilic blast cells (arrowed) within the thymic epithelium. H&E stained. $\times 450$. **C.** Autoradiograph of section of the E15 bursa 24 hrs after injection showing a labeled cell (arrowed) in the epithelium at a very early stage of bud formation. H&E stained. $\times 450$. **D.** Autoradiograph of section of the E15 bursa 24 hrs after injection showing labeled blast cells (arrowed) within and adjacent to an epithelial bud. H&E stained. $\times 450$.

gous to the para-aortic and dorsal aortic region of birds, were transplanted into triploid hosts. Cells from both sources entered the thymus at day 4 post-fertilization and contributed to thymocyte populations from the earliest developmental stage examined (9). Both VBI and DSC-derived cells persisted after metamorphosis and contributed to all lymphohematopoietic populations of the adult frog. In synchorial heterozygotic twin marmosets there is extensive thymic as well as bone marrow chimerism identified by sex chromosome analysis (21), supporting the concept that colonization of the developing thymus by immigrant cells occurs in all vertebrate species.

Initial colonization of the thymic rudiment precedes the onset of intrathymic vascularization by two days and is marked by accumulation of basophilic blast cells in perithymic mesenchymal capillaries, followed by their

egress and migration into contact with the epithelial anlage. Cytoplasmic extensions penetrate the epithelium basement membrane, and blast cells move into the epithelium and commence proliferation (Fig. 2). Intravenous injection of day 7 yolk sac cells labeled with tritiated thymidine into day-7 chick embryos show a comparable homing to thymic epithelium (Fig. 3). In the Nude mouse, lymphoid precursors fail to enter the epithelium from the perithymic mesenchyme, and the defect, which is in epithelial development, has been attributed to a loss-of-function mutation in the *Foxn1* transcription factor (109). MHC class II⁺ epithelial cells are a source of chemoattractant factors, and alymphoid thymic lobes can be shown to attract cells from fetal liver fragments (110).

While thymic epithelium plays an important role in T

cell development, less attention has been given to thymic mesenchyme. Mesenchyme surrounding the thymus is derived from cells that migrate from the cephalic region of the neural crest. The importance of this mesenchyme is demonstrated in studies in which E12 thymic lobes were cultured in the presence or absence of perithymic mesenchyme with the former generating all T cell subsets in vitro, whereas the latter showed severely compromised lymphopoiesis (26). Earlier studies had suggested that the mesenchyme influenced thymic epithelial morphogenesis and the effects on lymphopoiesis were secondary to a defective epithelium; however, there is clear evidence that mesenchymal cells directly affect lymphopoiesis (111). Mesenchymal cells migrate into the epithelial thymus and establish an intrathymic network of fibroblasts with an associated extracellular matrix (ECM). ECM components such as fibronectin and laminin interact with integrins expressed on developing thymocytes, initiating outside-in signaling, and sulfated glycosaminoglycans in the matrix can bind cytokines and chemokines which may be presented to thymocytes. Members of the BM and fibroblast growth factor (FGF) family of growth factors have been implicated in cross talk between stromal cells and the thymocyte compartment (112,113). Impaired thymic development is seen in mice lacking either FGF10 or its receptor FGFR2IIIb (112). The ligands for this receptor (FGF7 and FGF10) are synthesized by thymic mesenchyme, while the receptor is expressed on thymic epithelium. BMP4 acts upstream of FGF7/FGF10, to upregulate Foxn1, leading in turn to upregulation of FGFR2IIIb on epithelium (113). BMP4 also upregulated the stromal chemokines eotaxin and EB-11 ligand (ELC), in an FGF signal-dependent manner (113). Using thymic organ culture, both BMP4 and FGF7/FGF10 arrested early T cell development at the CD4-CD8-CD25-CD44⁺ (DN1) stage and at the CD4-CD8- double negative (DN) to CD4+CD8+ double-positive (DP) transition in a stromal compartment-dependent manner (113).

ARE THYMIC COLONIZING CELLS LYMPHOMYELOID STEM CELLS (HSC), COMMON LYMPHOID PROGENITORS (CLP) OR T LINEAGE-RESTRICTED PROGENITORS (PT)

Limiting dilution in vitro and in vivo thymic lobe colonizing assays have been used to identify thymic repopulating cells and can be used in parallel with B cell precursor assays in stromal co-culture, myeloid progenitor colony assays, or HSC detected by competitive long-term repopulating assays. Lymphoid commitment has been reported to precede thymic development in the CD45+cKit+CD34+ hematopoietic clusters of the vascular endothelium of the AGM region (72, 114–118).

In addition to multilineage progenitors, progenitors restricted to T lineage (pT) or T, NK and macrophage lineages (pTM), or B and B plus macrophage lineage (pBM) were identified at this early stage. The common lymphoid progenitor (CLP), identified in adult mouse marrow as a precursor of both B and T lineage and possibly involved in colonization of the post-natal thymus (119), does not exist in the embryo (114,120–122). The AGM pT cells have TCR rearrangements and are subsequently found in the circulation and fetal liver at E11–12, coinciding with thymic colonization (122). In the mouse embryo ~20 T cell precursors enter the thymus at E11–12, increasing to a maximum of ~3,000 entering between E13–14 (123). This is compatible with the number of CD45⁺ cells in each E12 thymus lobe (~50 cells) (124), and the 100–200 large basophilic cells within the epithelium and the 90–100 in the perithymic mesenchyme reported between E12–E13 (17). At the time of entry into the thymic epithelium at E12, the immigrant cells express CD45⁺, c-kit⁺, CD44⁺, CD34⁺ and the α_4 integrin, but are negative for CD62L, CD25 and Thy-1.2 (124). CD34 is downregulated on thymic precursors between 24–48 hrs after entry and so is a marker for the earliest thymic stem cell in the embryo. The CD45⁺ migrant stem cells can still be seen in the mesenchyme surrounding the epithelium at E13, suggesting the first wave of stem cells is still entering, but by E14, stem cells are no longer seen in the perithymic connective tissue (124).

It is possible to isolate thymic immigrant cells from the perithymic mesenchyme that have exited the circulation, but prior to their exposure to epithelial inductive influences. These cells are IL7 α ⁺ and do not yet express Notch target genes such as *Hes-1* and *pre-T α* , but upon entry into the thymic epithelium and exposure to Notch ligands Delta and Jagged, Notch signaling is activated (125). Ectopic expression of Delta ligand on a marrow stromal line, in the presence of IL7 ligand and cKit ligand reproduced a thymic inducing environment that supported differentiation of fetal liver Sca-1^{hi} c-Kit^{hi} CD24^{lo}Lin-progenitors to functionally mature CD4 and CD8 T cells (126). IL7 α appears to be downregulated on the earliest T lineage progenitor within the thymus associated with loss of thymic homing capacity (127). Mouse IL7 α chain mutants show profound defects in thymopoiesis in the adult, but fetal thymopoiesis is relatively intact, suggesting possible alternate cytokine pathways can operate during ontogeny (128).

The first 14 days of thymic development in the mouse can be separated into four phenotypically and genetically distinct phases (129). The first stage (lineage double negative stage 1) is CD4-CD8-CD25-CD44^{hi} and initially CD34⁺ gives rise to T, B, dendritic cell (DC) and NK; the second stage is CD4-CD8-CD25+CD44^{hi} T or DC only. Stage 3 are CD4-C8-CD25+CD44^{lo} and absolutely committed to T. The final stage is pre-CD4+CD8+CD25.

A 4000-fold expansion occurs in the first 3 phases and a 250-fold expansion in the early pre-double positive stage.

DEVELOPMENT OF B LYMPHOCYTES IN THE AVIAN BURSA OF FABRICIUS

B lymphopoiesis is initiated in avian species in the bursa of Fabricius—an organ uniquely specialized for this purpose. The search for the mammalian equivalent of the bursa has led to various sites as being proposed as “bursal equivalent,” even the appendix, but it is most likely that mammalian B lymphopoiesis occurs in the fetal liver before moving to the bone marrow. The bursal primordium appears by E5 as an epithelial thickening at the ventro-caudal contact of the cloaca with external ectodermal epithelium. By E10–11 longitudinal folds of lining epithelium project into the lumen, and by E12–13 epithelial buds project into the underlying mesenchyme (Fig. 4). Studies in parabiosed embryos and embryonic grafts of bursal rudiments demonstrated that the bursa was colonized by circulating lymphoid precursors that entered at E13–14 (5, 14, 15). At this stage large basophilic blast cells are observed in the blood vessels of the mesenchyme adjacent to the epithelial buds. After exiting the vessels the blast cells migrate into contact with the epithelial buds before transiting through the epithelial basement membrane (Fig. 4) and localizing within the epithelium follicle where they proliferate extensively, differentiating into typical bursal B lymphocytes (5, 17). Injection of embryos at E10–14 with tritiated thymidine-labeled embryonic yolk sac, spleen marrow, and blood cells (but not thymocytes) showed rapid localization of labeled basophilic cells in the bursal epithelium with subsequent proliferation and lymphoid differentiation (Fig. 3). Chromosome marker studies showed that injection of the same embryonic hematopoietic tissues, but not embryonic thymus, into E14 irradiated embryos confirmed their ability to reconstitute bursal lymphopoiesis (15). Cells present in the E14 spleen or marrow that colonized the bursa upon injection were already B committed with VJ recombination, and distinct from T lineage precursors (130). The precise site of B cell commitment is not known, but marker studies indicate that the B precursors ultimately derive from the intraembryonic para-aortic region, possibly as early as E2 (39, 40), although their subsequent expansion and B commitment may occur within the yolk sac and then spleen.

THE MAMMALIAN LIVER AS THE MAJOR SITE OF FETAL HEMATOPOIESIS AND B CELL DEVELOPMENT

The liver develops from ventral foregut endoderm that is induced to proliferate and adopt a hepatic fate by sig-

nals from pericardium and septum transversum mesenchyme (131). In studies involving grafting of fetal liver beneath the adult mouse kidney capsule we showed that autonomous hematopoiesis occurred if the graft was from embryos older than 28 somites (E10), whereas grafts of earlier stages did not show hematopoiesis (36). Injection of hematopoietic cells intravenously into irradiated, fetal liver-engrafted mice showed that the pre-28 somite graft could support hematopoiesis. This study established E9.5–10 as the time of initial colonization of the fetal liver by immigrant stem/progenitor cells. Limiting dilution long-term repopulation assays demonstrate at least one competitive repopulating unit (CRU) in the liver, circulation, AGM region, and YS at E11, with an increase in the liver of >50-fold by E12 and >250-fold at E13 (43). After correcting for in vivo seeding efficiency (~10%) the data suggests an initial homing of 10 stem cells at E10–11. The >250-fold expansion of stem cells in the ensuing 48 hrs is matched by a daily logarithmic increase in CFU-GM BFU-E and CFU-S between E10–13 (17, 35). It is likely that continual immigration of stem and progenitor cells occurs with initial colonization from the AGM, but at E12 the YS is making a significant contribution (43). Large numbers of CFU-E appear simultaneously with BFU-E at the onset of hepatic hematopoiesis, and embryonic blood contains significant numbers of definitive hematopoietic progenitors immediately prior to liver development indicating colonization by immigrant progenitors in addition to stem cells (56). A derivation from YS is suggested by studies in human fetuses showing that BFU-E numbers drop abruptly in the YS at 35 days and simultaneously appear in the liver, reflecting oriented migration (47). Thus the liver appears to be colonized by two waves of HSC, the initial one from the AGM arriving at E10, reaching a maximum at E11 and disappearing by E13, and a second wave arriving from the YS on E12. Both these waves are accompanied by immigration of multipotent and unipotent progenitors and possibly macrophages, predominantly of YS origin.

The fetal liver has long been considered the initial site of B cell commitment beginning at E14 in the mouse, and from then on, the B progenitors expand in a synchronous wave-like pattern reaching a peak in the perinatal stage (132). By functional and phenotypic analysis AA4.1⁺, FcγR⁺ or AA4.1⁺, Sca-1⁺, B220^{-ve} cells with B/macrophage potential are detected in E12.5–13 fetal liver (99, 133–136). A novel population of cKit⁺, AA4.1⁺, CD34⁺, Sca-1^{-ve}, CD45⁺, C19⁺ cells (70% expressing IL7Rα but negative for CD45R/B220) have been reported in E11 AGM and liver that differentiate exclusively into B cells (135). Numerically at E11 these very early pro-B progenitors comprised ~100 in the AGM region, ~500 in the liver, with smaller numbers in the blood and yolk sac. Migration of these B progenitors from the AGM to the liver begins at E10 with subsequent increase due to con-

FIG. 4. E14 chick embryo bursa of Fabricius. At this stage the epithelium is budding into the mesenchyme of the tunica propria and chromosome marker studies of parabiosed embryos shows this time to be the onset of bursal chimerism. **A.** A cluster of basophilic blast cells in a blood vessel in the perifollicular mesenchyme. Note a blast cell extruding pseudopodia, apparently traversing the endothelial lining (arrowed) and a blast cell in the mesenchyme Giemsa stain. $\times 1000$. **B.** Basophilic blast cells in and around an epithelial bud. A basophilic pseudopod can be seen extending across the epithelial basement membrane from a cell outside the plane of section (arrowed). $\times 750$. **C.** A circumscribed epithelial follicle containing a large basophilic blast cell, and a pair of smaller basophilic cells, probably a division product of an immigrant blast cell. $\times 750$. **D.** An E18 bursal follicle from an embryo treated with colcemid to block cells in metaphase (small dark nuclei). Note the predominant lymphoid morphology and high mitotic rate. $\times 250$.

tinued immigration and intra-hepatic expansion. Since HSC are also colonizing the liver at this time, the possibility of intra-hepatic generation of additional pB from these more primitive cells cannot be excluded. The myeloid suppressing transcription factor Pax5 plays a critical role in B cell commitment, and Pax5-deficient mice display B cell maturation arrest at the pro-B level (137). The B/macrophage progenitors in the fetal liver and AGM expresses Pax5 but at a significantly lower level than the adult CLP (136). This observation may account for the retention of macrophage potential in the fetal B progenitor and complete block of this pathway in the adult CLP. The pB of Pax5-deficient mice provide long-term reconstitution of the thymus of RAG2-deficient mice and generate mature T cells, and in vitro with appropriate cytokines, can produce T cells, macrophages, osteoclasts, dendritic cells, granulocytes, and NK cells (137).

The role of the chemokine SDF-1 in promoting the migration of pB from the AGM region to the liver is suggested by the demonstration that its receptor, CXCR4, is expressed on $IL7R\alpha^+$ pB and SDF-1 induces chemotaxis of these cells (138). Mice with targeted disruption of the SDF-1 or CXCR4 genes display defects in B lymphocyte development in the fetal liver (139,140) that may be attributed to defective colonization of the liver by pB as well as impairment in B cell proliferation, since SDF-1, acting in synergy with cKit and Flt3 ligands, promotes proliferation of pB cells (138). The numbers of HSC (CRU) were normal in fetal liver of SDF-1 null embryos between E12.5–E14.5 with a two-fold reduction at E16.5 but normal numbers by E18.5 (141). This contrasted with severe depletion of HSC in E18.5 BM indicating a critical role for SDF-1 in stem cell homing to marrow but not to fetal liver.

SPLEEN DEVELOPMENT

The spleen develops as a hematopoietic organ at E13 in the mouse, prior to bone marrow development. Splenic hematopoiesis is initiated at E8 in chick embryos. The splenic primordium appears as a dense syncytial-like mesenchymal thickening in the dorsal mesogastrium interspersed with vascular spaces. Chromosome marker studies in parabiosed and twin chick embryos demonstrated extensive chimerism in the splenic rudiment as early as E12 (14). Cell labeling studies confirmed that circulating cells colonized the chick splenic rudiment as early as E8 (17), and together with embryonic hematopoietic cell reconstitution of irradiated embryos, indicated that splenic colonizing cells were present in the YS and circulation at the time of initiation of splenic hematopoiesis (15). The onset of cell immigration into the developing spleen coincides with the appearance of large immature cells characterized by intense cytoplasmic basophilia and prominent nucleoli both in the vascular spaces and perivascular mesenchyme of the spleen (15). In the chick embryo by E9 these basophilic cells appear scattered throughout the mesenchyme and frequently extend long tails of cytoplasm between the reticulum cells for some distance from the main body of the cells. E11 granulopoiesis is extensive and erythropoietic foci develop. In mammals, the fetal liver is the most probable source of cells initially colonizing the spleen rudiment since the AGM and yolk sac are no longer hematopoietic at this stage (E13–14), and the marrow at this early stage is a net importer rather than exporter of cells. CFU-GM/BFU-E, CFU-s and HSC are detected by E15 in the murine splenic rudiment and increase in absolute numbers through to 3 weeks post-natally, then progressively decline as the spleen ceases to be a myelopoietic organ (17). Labeled thymic lymphocytes began to localize in the spleen with high efficiency by E17 coinciding with the initiation of lymphopoiesis. In birds and mammals splenic myelopoietic activity progressively declines and the spleen becomes a predominantly lymphopoietic organ.

BONE MARROW DEVELOPMENT

Bone marrow development begins by penetration of perichondrial mesenchymal cells and blood vessels into the zone of calcified cartilage in the central region of the long bones (142). Vascular cells are recruited by VEGF secreted by hypertrophic chondrocytes and bring along osteoblast precursors and circulating hematopoietic cells, including primitive macrophages (143). The cartilaginous matrix is resorbed and the primordial marrow cavity appears as a mesenchymal network interspersed with a plexus of widely dilated veins. Hematopoiesis is initiated

by accumulation of large numbers of undifferentiated basophilic blast cells within the dilated marrow capillaries, beginning at E11–12 in chick, E17 in mouse, and E70–77 in man (17). Avian parabiosis studies demonstrated that circulating stem cells and progenitors colonize the developing marrow beginning at E11–12 (14). Following intravenous injection of tritiated thymidine-labeled embryonic YS or spleen cells into chick embryos, significant numbers of labeled blast cells localized in the marrow at E11 (17). Cells from these tissues also repopulated the marrow when injected into irradiated embryos (15). In the mouse, CFU-GM/BFU-E and CFU-s and HSC appear in the femoral marrow at E17, and the populations double every 34 hrs.

Evidence that fetal liver was the source of stem/progenitor cells for colonizing the marrow came from studies in which rats were injected in utero at E16 with a retroviral vector (144). Clonal identification of viral integration sites showed that fetal liver-derived clones appeared in the marrow and circulated throughout the life of the animals. The role of SDF-1 produced by marrow stromal cells in the chemoattraction of CXCR4⁺ HSC is well established in the adult (145), and the failure of development of marrow hematopoiesis in mice with inactivation of the CXCR4/SDF-1 pathway (139,140) strongly suggests that the initial wave of HSC migration is also SDF-1 dependent. In the marrow of embryonic SDF-1^{-/-} mice at E18.5 the numbers of HSC, Gr-1⁺ (myeloid), and B220/CD19⁺ B lymphoid cells were severely reduced, but numbers of Ter119⁺ erythroid cells were normal or only modestly reduced (141). SDF-1 in the mesenchymal cells adjacent to endothelial cells appears to play a critical role in colonization of BM by HSC and myeloid and lymphoid progenitors. In rodents, the marrow progressively expands in the first two months of postnatal life, associated with a decline in hepatic hematopoiesis in the first week of life and a decline of splenic hematopoiesis after the third week.

CONCLUSIONS

The use of various cell marker systems has revealed the dynamic nature of cell migration between developing hematopoietic and lymphoid organs. Hematopoietic stem cells with lymphomyeloid potential are the most likely candidates for cells trafficking from the YS and AGM to fetal liver, spleen, and bone marrow, while lymphoid restricted progenitors appear more likely for primary lymphoid organ colonization. The long debate over the respective roles of YS and AGM in generating the HSC for subsequent hematopoietic development is near a resolution since definitive HSC capable of colonizing the fetal liver first arise in the yolk sac while HSC capable of re-

populating adult animals first arises in AGM and shortly thereafter in YS, with both sources participating in liver colonization. A central theme of these developmental observations is that stem cells (and in lymphoid pathways, B or T restricted progenitor cells) traffic from their sites of origin to specific niches within other organs that support expression of specific differentiation programs. These niches may be at sites of endodermal and mesodermal interaction (yolk sac, thymus, bursa, fetal liver), or be of exclusively mesodermal origin (spleen, marrow). This highly organized system of temporal migration streams of stem/progenitor cells moving through the circulation, and entering appropriate inductive environments seems the antithesis of the emerging concepts of cell plasticity in which hematopoietic stem cell-derived populations can switch not only lineage but also germ layer specificity.

Plasticity has been defined as the ability of adult stem cells to cross lineage barriers and adopt the expression profile and functional phenotype of cells that are unique to other tissues (146). Bone marrow has been identified as a source of cells as diverse as neurons and glia, lung bronchiolar epithelium, and type II pneumocytes, skeletal and cardiac muscle, keratinocytes, intestinal epithelium, hepatocytes, pancreatic beta cells, renal epithelium, mesangial cells, and interstitial cells (reviewed in ref. 146). A number of explanations can be evoked to explain these remarkable observations and these need not all be mutually exclusive. The first may be that some of the observations are erroneous for various technical or methodological reasons, and the irreproducibility of at least some of the data supports this view. Secondly, the marrow may harbor many different types of stem cell. There is no argument about the existence of hematopoietic stem cells, mesenchymal stem cells, and endothelial stem cells within adult marrow (and fetal liver), and it is possible that certain unique features of the marrow provide appropriate niches for the long-term maintenance of many different types of stem cells. These niches may protect stem cells from differentiation pressure to which they are subject once they egress the marrow and home to appropriate target sites, e.g., to muscle or sites of neovascularization in the case of endothelial stem cells. More primitive stem cells may also reside in the marrow, albeit at very low frequencies. Hemangioblasts may persist from early developmental stages since there have been studies where single cell cloning or engraftment experiments have suggested their presence in adult marrow (147–149). While most CD34+, CD133+ marrow cells expressing VEGFR2/KDR/Flk-1 are endothelial progenitor/stem cells, a low percentage of these cells may give rise to hematopoiesis and endothelium (149).

The intimate association between hematopoietic stem cells and endothelium (150) including possibly emperipolesis, protein interchange (151), and cell fusion,

does raise concerns about artefacts in this type of study. Perhaps the most remarkable marrow stem cell (or more correctly a stem cell derived by long-term in vitro culture of a CD45-ve marrow population enriched for mesenchymal stem cells under low cell density and highly specific culture conditions), is the Multipotential Adult Progenitor Cell (MAPC) (152,153). These cells can be differentiated in vitro into endodermal, ectodermal, and mesodermal lineages. Murine MAPC when injected into blastocysts contributed to virtually all somatic tissue of the chimeric mice (153). Transplantation of MAPC into sublethally irradiated immunodeficient mice revealed their potential to form CD45+ hematopoietic cells as well as epithelial cells in lung, liver, and intestine. The phenotype and gene expression profile of MAPCs suggest a very primitive cell closer to the primordial germ cell or embryonic stem cell than to either hematopoietic or mesenchymal stem cells. It shares with germ line cells and ES cells the presence of high levels of telomerase, conferring extensive, potentially unlimited, proliferative potential without telomere erosion. The development origins of MAPCs are unknown, if indeed they are normally present in marrow. They may be artificially created by some process of dedifferentiation of mesenchymal stem cells during the prolonged in vitro culture required for their isolation. If they are not some artefact, then they may have some developmental relationship to cells of the inner cell mass that generate ES cells, or the primordial germ cells that are trafficking through the developing embryo at the same time as hematopoietic stem cells are trafficking from yolk sac and AGM to the fetal liver.

If indeed MAPCs bear some developmental relationship to these developmentally primitive, pluripotent stem cells, they should be detected at some stages of development, within the circulation, either trafficking to the fetal liver in early development, or from the liver to the bone marrow at later stages of development. The MAPC has the potential to generate hematopoietic, endothelial, and mesenchymal stem cells and therefore could potentially migrate to a site, e.g., the developing bone, and generate all components of a hematopoietic organ. However, there is no evidence that such a cell plays any role in hematopoietic organogenesis since the evidence points to the local origin of mesenchymal stem cells from the local mesoderm with their differentiation to stroma (154–156) and to osteoblasts which create a critical stem cell-supportive niche for immigrant hematopoietic stem cells (160–161).

A third explanation for plasticity is that a process of transdifferentiation occurs with low frequency, in which a lineage committed cell changes its gene expression pattern to that of a completely different type. A variant of this is that a cell undergoes dedifferentiation and reprogramming along another lineage. Certainly such a pro-

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cess occurs during somatic nuclear transfer to the enucleated oocyte in reproductive and therapeutic cloning. It is probably also occurring during somatic cell fusion that has been shown to occur in a number of experimental systems. Neural stem cells and bone marrow cells have been shown to fuse with ES cells and take on ES properties (157,158). The marrow-derived fusions formed clones that were either tetraploid or hexaploid, and these could form embryoid bodies that differentiated into multiple tissues (159). Fusion was not obtained with purified HSC and it was suggested that fusion involved macrophages (159). The significance of cell fusion has been revealed in a novel transplant system in which marrow was transplanted from mice with Cre recombinase knocked into the CD45 locus promoter and transplanted into a conditional Cre reporter mouse line R26R with the LacZ reporter gene exclusively expressed after excision of the loxP (floxed) stop cassette by Cre-mediated recombination (160). LacZ expressing cells were identified in liver, brain (Purkinje cells), and heart indicating that fusion between hematopoietic-derived cells and differentiated cells occurs in these sites. Cell fusion has been shown to be responsible for hepatic regeneration in other recent studies (161,162) and the intimate association of Kupffer cells of the macrophage lineage with hepatocytes suggests that they are likely the fusion candidates. However, fusion cannot account for all instances of "plasticity" and the in vivo derivation of glucose-competent pancreatic endocrine cells from mouse bone marrow was demonstrated in a system that excluded the possibility of cell fusion (163).

In my forty years of research into the hematopoietic stem cell and its properties, I have seen the "black box" that we labeled "the stem cell" gradually reveal its contents. The evolution of scientific ideas, certainly in cell biology, is generally marked by an initiating concept followed by an oversimplified premature conclusion, followed in turn by a period of anarchy with conflicting interpretations, until finally a consensus is arrived at involving models inherently more complex than initially thought. I would like to believe that we are entering this final phase.

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