

CHAPTER 1

Stem cells and haemopoiesis

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Introduction

Haemopoietic stem cells (HSCs) are the foundation of the adult blood system and sustain the lifelong production of all blood lineages. These rare cells are generally defined by their ability to self-renew through a process of asymmetric cell division, the outcome of which is an HSC and a differentiating cell. In health, HSCs provide homeostatic maintenance of the system through their ability to differentiate and generate the hundreds of millions of erythrocytes and leucocytes needed each day. In trauma and physiological stress, HSCs ensure the replacement of the lost or damaged blood cells. The tight regulation of HSC self-renewal ensures the appropriate balance of blood cell production. Perturbation of this regulation and unchecked growth of HSCs and/or immature blood cells results in leukaemia. Over the last 50 years, great success has been achieved with bone marrow transplantation as a stem cell regenerative therapy. However, insufficient numbers of HSCs are still a major constraint in clinical applications. As the pivotal cells in this essential tissue, HSCs are the focus of intense research to: (1) further our understanding of their normal behaviour and the basis of their dysfunction in haemopoietic disease and leukaemia and (2) provide insights for new strategies for improved and patient-specific stem cell therapies. This chapter provides current and historical information on the organization of the adult haemopoietic cell differentiation hierarchy, the ontogeny of HSCs, the stromal microenvironment supporting these cells, and the molecular mechanisms involved in the regulation of HSCs.

Hierarchical organization and lineage relationships in the adult haemopoietic system

The haemopoietic system is the best-characterized cell lineage differentiation hierarchy and, as such, has set the paradigm for the growth and differentiation of tissue-specific stem cells. HSCs are defined by their high proliferative potential, ability to self-renew and potential to give rise to all haemopoietic lineages. HSCs produce immature progenitors that gradually and progressively, through a series of proliferation and differentiation events, become restricted in lineage differentiation potential. Such restricted progenitors produce the terminally differentiated functional blood cells.

The lineage relationships of the variety of cells within the adult haemopoietic hierarchy (Figure 1.1) are based on results of *in vivo* transplantation assays in irradiated/myeloablated recipient mice and many *in vitro* differentiation assays that became available following the identification of haemopoietic growth factors. These assays facilitated measurement of the maturational progression of stem cells and progenitors, at or near the branch points of lineage commitment. Clonal analyses, in the form of colony-forming unit (CFU) assays or single cell transplantation assays, were developed to define the lineage differentiation potential of the stem cell or progenitor, and to quantitate the number/frequency of such cells in the population as a whole. In general, the rarer a progenitor is and the greater its lineage differentiation potential, the closer it is in the hierarchy to the HSC. *In vitro* clonogenic assays measure the most immature

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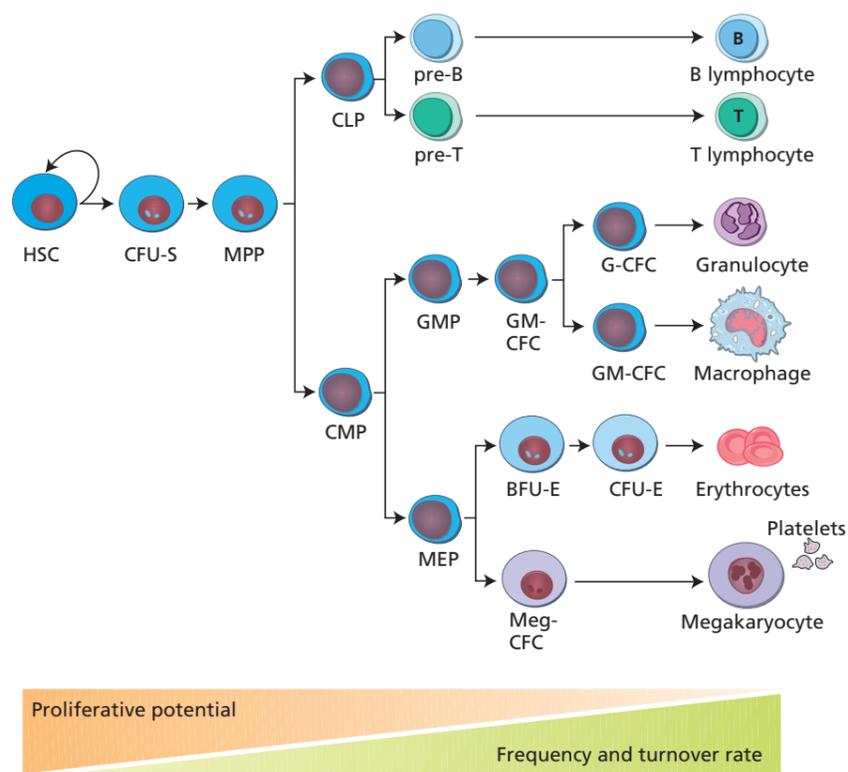


Figure 1.1 The adult haemopoietic hierarchy. Haemopoietic stem cells are at the foundation of the hierarchy. Through a series of progressive proliferation and differentiation steps the mature blood cell lineages are produced. Haemopoietic stem cells have the greatest proliferative and multilineage differentiation potential, while the mature blood cells are not proliferative and are lineage restricted. While large numbers of mature cells are found in the blood and turn over rapidly, the bone marrow contains long-lived quiescent haemopoietic stem cells at a very low frequency.

progenitor CFU-GEMM/Mix (granulocyte, erythroid, macrophage, megakaryocyte), bipotent progenitors CFU-GM (granulocyte, macrophage) and restricted progenitors CFU-M (macrophage), CFU-G (granulocyte), CFU-E (erythroid) and BFU-E (burst-forming unit-erythroid). While such *in vitro* clonogenic assays measure myeloid and erythroid potential, lymphoid potential is revealed only in fetal thymic organ cultures and stromal cell cocultures in which the appropriate microenvironment and growth factors are present. Long-term culture assays (6–8 week duration), such as the cobblestone-area-forming cell (CAFC) and the long-term culture-initiating cell (LTC-IC) assays, reveal the most immature of haemopoietic progenitors. Currently, the major hurdle in studies and clinical applications of HSCs is the fact that HSCs cannot be expanded and are poorly maintained in culture. The only way to detect a *bona fide* HSC is *in vivo*.

In vivo, the heterogeneity of the bone marrow population of immature progenitors and HSCs is reflected in the time periods at which different clones contribute to haemopoiesis. Short-term *in vivo* repopulating haemopoietic progenitor cells such as CFU-S (spleen) give rise to macroscopic erythro-myeloid colonies on the spleen within 14 days of injection. *Bona fide* HSCs give rise to the long-term high-level engraftment of all haemopoietic lineages. Serial transplantations reveal the ability

of the long-term repopulating HSCs to self-renew. The clonal nature of engraftment and the multilineage potential of HSCs has been demonstrated through radiation, retroviral and barcode marking of bone marrow cells. Such studies suggest that, at steady state, several HSC clones contribute to the haemopoietic system at any one time. Further analyses of bone marrow HSCs show that this compartment consists of a limited number of distinct HSC subsets, each with predictable behaviours, as described by their repopulation kinetics in myeloablated adult recipients. In general, the bone marrow haemopoietic cell compartment, as measured by *in vitro* clonogenic assays and *in vivo* transplantation assays, shows a progression along the adult differentiation hierarchy from HSCs to progenitors and fully functional blood cells with decreased multipotency and proliferative potential.

The use of flow cytometry to enrich for HSCs and the various progenitors in adult bone marrow has been instrumental in refining precursor–progeny relationships in the adult haemopoietic hierarchy. HSCs are characteristically small ‘blast’ cells, with a relatively low forward and side light scatter and low metabolic activity. Both mouse and human HSCs are negative for expression of mature haemopoietic lineage cell-surface markers, such as those found on B lymphoid cells (CD19, B220), T lymphoid cells (CD4, CD8, CD3), macrophages (CD15,

Mac-1) and granulocytes (Gr-1). Positive selection for mouse HSCs relies on expression of Sca-1, c-kit, endoglin and CD150 markers and for human HSCs on expression of CD34, c-kit, IL-6R, Thy-1 and CD45RA markers. Similarly, cell types at lineage branch points have been identified, including the CMP (common myeloid progenitor), CLP (common lymphoid progenitor) and GMP (granulocyte macrophage progenitor). Recently, using the Flt3 receptor tyrosine kinase surface marker along with many other well-studied markers, the LMPP (lymphoid primed multipotent progenitor) has been identified within the lineage negative, Sca-1 positive, c-kit positive (LSK) enriched fraction of HSCs. These cells have granulocyte/macrophage, B lymphoid and T lymphoid potential, but little or no megakaryocyte/erythroid potential. This suggests that the first lineage differentiation event is not a strict separation into common lymphoid and myeloid pathways. While these cell-surface marker changes and functional restriction events are represented by discrete cells in the working model of the haemopoietic hierarchy as depicted in textbooks and Figure 1.1, it is most likely that there is a continuum of cells between these landmarks and/or alternative differentiation paths. The currently identified progenitor cells in the hierarchy represent the cells present at stable and detectable frequencies and for which we currently have markers and functional assays. As more cell-surface markers are identified and the sensitivity of detection is increased, additional intermediate cell subsets are likely to be identified. Together with single cell transcriptomic approaches, it may be possible to predict the molecular events needed for the HSC state and the differentiation of the entire haemopoietic system.

Sites of adult haemopoiesis

Bone marrow, spleen, thymus and lymph nodes are the haemopoietic sites in the adult, and each tissue plays a special role in supporting the growth and differentiation of particular haemopoietic cell lineages and subsets. Equally important is the blood itself, which is a mobile haemopoietic tissue, with mature blood cells travelling through the circulation to function in all parts of the body. Not only do the terminally differentiated cells, such as erythrocytes and lymphocytes, move by means of the circulation, but HSCs (at low frequency) also migrate through the circulation from the bone marrow to other haemopoietic tissues. HSCs are mostly concentrated in the bone marrow and are found in the endosteal and vascular niches (Figure 1.2). HSCs can be induced to circulate by administration of granulocyte colony-stimulating factor (G-CSF). Recent improvements in confocal microscopy have allowed the visualization of the migration of circulating HSCs to the bone marrow endosteal niche by time-lapse imaging in the mouse.

The estimated frequency of HSCs is 1 per 10^4 – 10^5 mouse bone marrow cells and 1 per 20×10^6 human bone marrow cells.

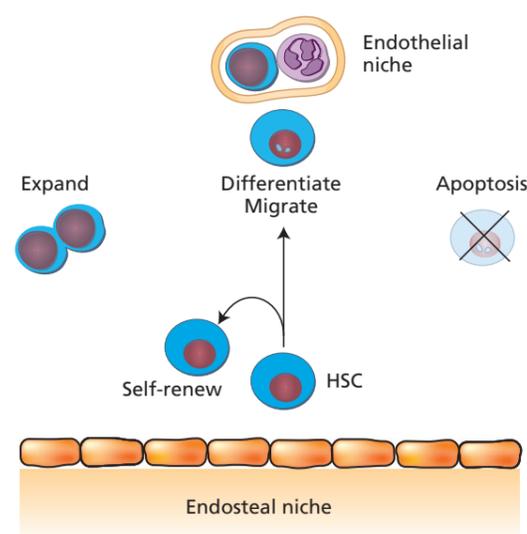


Figure 1.2 The bone marrow haemopoietic niches. Haemopoietic stem cells are found in the endosteal and endothelial niches of the bone marrow. These niches support the maintenance, self-renewal, expansion, differentiation, migration and survival of haemopoietic stem cells through local growth factor production and cell–cell interactions.

HSCs are also found in the mouse spleen at approximately a 10-fold lower frequency and in the circulating blood at a 100-fold lower frequency. The capacity for HSCs to migrate and also be retained in bone marrow supportive niches is of relevance to clinical transplantation therapies. HSCs injected intravenously in such therapies must find their way to the bone marrow for survival and effective haemopoietic engraftment. For example, stromal-derived factor (SDF)-1 and its receptor CXCR4 (expressed on HSCs) are implicated in the movement of HSCs and the retention of HSCs in the bone marrow. Indeed, HSC mobilization can be induced through AMD3100, an antagonist of SDF-1, and by the administration of G-CSF. Mobilization strategies with G-CSF are used routinely to stimulate bone marrow HSCs to enter the circulation, allowing ease of collection in the blood rather than through bone marrow biopsy.

Development of HSCs

Waves of haemopoietic generation in embryonic development

Until the mid-1960s it was thought that blood cells were intrinsically generated in tissues such as the liver, spleen, bone

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marrow and thymus. Survival studies in which cells from un-irradiated tissues were injected into lethally irradiated mice showed that it was the bone marrow that contains the potent cells responsible for rescue from haemopoietic failure. Later, through clonal marking studies, it was demonstrated that the bone marrow harbours HSCs during the adult stages of life. But where, when and how are HSCs generated during ontogeny? In the 1970s, examination of mouse embryo tissues suggested that adult haemopoietic cells are generated in the yolk sac, migrate and colonize initially the fetal liver and subsequently the bone marrow, where they reside throughout adult life. However, studies in non-mammalian vertebrate models (avian and amphibian) demonstrated that the aorta region in the body of the embryo generates the long-lived adult blood system, while the yolk sac (or equivalent tissue) produces the transient embryonic haemopoietic system. In agreement with these studies, the aorta-gonad-mesonephros (AGM) region of mammalian embryos was later found to generate the first HSCs of the permanent adult blood system.

The development of the haemopoietic system is complex. As a growing organism, the embryo itself needs rapid haemopoiesis to allow it to thrive before the adult system is generated. Thus, a simple transient haemopoietic system is generated at early embryonic stages to rapidly produce primitive erythroid and myeloid cells. In the yolk sac, both haemopoietic and endothelial cells are simultaneously generated from a common mesodermal precursor cell, the haemangioblast (Figure 1.3). Thereafter, other haemopoietic progenitor and differentiated cell types are generated in both the yolk sac and the intraembryonic AGM region to create an intermediate haemopoietic system. These progenitors and differentiated cell types arise from a specialized population of endothelial cells that have haemogenic potential (haemogenic endothelial cells). At both these early times in ontogeny, the mouse embryo contains no HSCs. Hence, in the absence of HSCs, the embryo generates a haemopoietic system that is short-lived and lacks the important qualitative character-

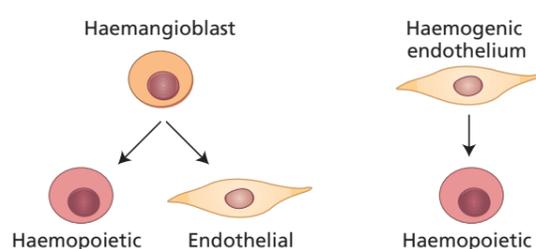


Figure 1.3 Precursors to haemopoietic cells in embryonic stages. The mesodermal precursor to haemopoietic and endothelial lineages at early stages of development is the haemangioblast. Later, haemogenic endothelial cells are the precursors to haemopoietic stem cells and progenitor cells. Both precursors appear to exist during a short window of developmental time.

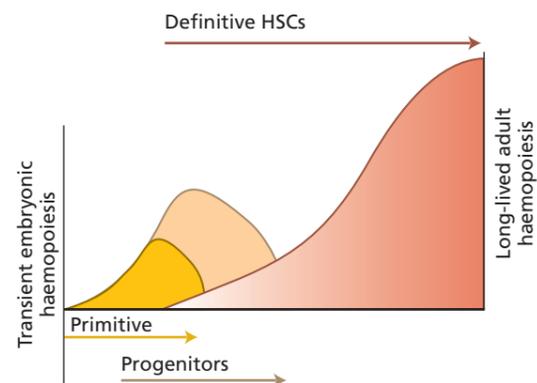


Figure 1.4 Waves of haemopoietic cell emergence during embryonic stages. The earliest haemopoietic cells are produced during the first wave of haemopoietic fate determination. The onset of this wave occurs in the yolk sac blood islands and produces transient primitive erythroid cells. This wave continues with the production of erythroid-myeloid progenitors in the absence of *bona fide* haemopoietic stem cells. True long-lived self-renewing definitive haemopoietic stem cells (adult repopulating stem cells) are generated in the second wave of haemopoietic cell emergence in the AGM region. In this wave, haemogenic endothelial cells bud into the aortic lumen as these cells take on haemopoietic stem cell fate.

istics (longevity and self-renewability) of the adult haemopoietic system. However, some early yolk sac progenitors provide long-lived tissue resident macrophages, such as the glial cells in the brain. The independent and distinct waves of haemopoiesis that supply the embryo and adult are likely derived from different subsets of mesodermal precursor cells (Figure 1.4).

The adult system has its foundation in a cohort of initiating HSCs. The first adult HSCs are autonomously generated in the mouse AGM at E10.5 and in the human AGM beginning at week 4 of gestation. Recently, the process of HSC generation has been visualized in real time in the mouse embryo. This remarkable demonstration confirms that HSCs are derived via a transdifferentiation event in which specialized endothelial cells lining the aorta bud into the lumen to form round cells with HSC fate (Figure 1.5), and shows that haemopoietic development is conserved between mammalian and non-mammalian species. The emerging mouse aortic HSCs are characterized by the loss of cell-surface markers for endothelium, such as Flk-1 and VE-cadherin, and the gain of expression of haemopoietic markers CD41 and CD45 and HSC markers Sca1, c-kit and endoglin. The emerging aortic HSCs are as functionally potent as bone marrow HSCs, since these sorted cells can form a complete long-term haemopoietic system and self-renewing HSCs after transplantation into irradiated adult recipient mice.

Lineage tracing experiments in the mouse embryo have indicated that the adult haemopoietic system is generated during a

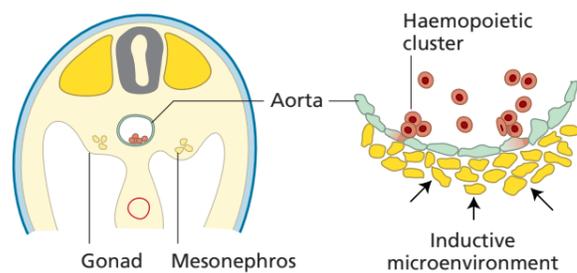


Figure 1.5 Schematic diagram of the aorta-gonad-mesonephros (AGM) region and haemopoietic cell clusters emerging from the dorsal aorta. The haemopoietic stem-cell-inductive microenvironment is localized in the ventral aspect of the aorta. Tissues ventral to the AGM, such as the gut and mesenchyme provide HSC-inducing signals, whereas dorsal tissues such as the notochord and the neural tube suppress HSC induction.

short window of development, spanning E9–E12. Using Cre-lox recombination (temporally and cell-lineage controlled) to mark endothelial cells in the mid-gestation embryo, it was found that almost all the blood cells in the circulation and haemopoietic tissues of the adult mice were derived from VE-cadherin expressing cells. Moreover, these cells require the Runx1 transcription factor, as demonstrated by Runx1 conditional deletion in this mouse model. Other lineage tracing experiments marking the earliest cells expressing the *Runx1* and *SCL* transcription factor genes, showed that the progeny contributed to the bone marrow cells in the adult. Thus, the progeny of haemogenic endothelial cells in the major vasculature of the embryo contribute to a cohort of adult bone marrow HSCs that form the foundation of haemopoiesis throughout adult life.

Embryonic haemopoietic sites and haemopoietic migration

The AGM and yolk sac are not the only sites where haemopoietic cells are found in the early conceptus. The placenta is a highly haemopoietic tissue and much like the early-stage yolk sac, the mouse placenta can produce erythro-myeloid progenitors. Embryos deficient for the *Ncx1* gene lack a heartbeat and circulation, and thus were used to study the origins of early haemopoietic progenitors. *Ncx1* deficient embryos were shown to contain erythro-myeloid progenitors in the yolk sac and placenta, demonstrating that these haemopoietic progenitors are generated by these tissues. Unfortunately, the embryos die before the onset of HSC generation at mid-gestation, precluding analysis of HSC production in the yolk sac and placenta. In normal embryos where the circulation is established between the embryo body and the extraembryonic tissues at E8.25, HSCs are detected in the placenta and yolk sac only beginning at E11, subsequent to the first HSC generation in the AGM at E10.5.

A recent study revealed the presence and generation of HSCs in the E10.5/E11 head vasculature through lineage marking. It remains uncertain whether the placenta (or the yolk sac) can generate HSCs *de novo* since there is no method at present by which cells can be uniquely marked in these developing tissues. Nonetheless, quantitative studies in which HSC numbers in each of these tissues was determined suggest that the AGM cannot generate all the HSCs that are found in the fetal liver (a tissue that harbours haemopoietic cells but does not generate them) and later in the adult bone marrow (Figure 1.6). Since the placenta at mid-gestation contains an abundance of HSCs, it is possible that this highly vascularized tissue generates HSCs from haemogenic endothelium and/or that the placenta is a highly supportive and proliferative microenvironment for AGM-derived HSCs.

The development of the haemopoietic system in the human conceptus closely parallels that in the mouse conceptus. Like the mouse placenta, the developing human placenta contains HSCs. Already at week 6 of gestation HSCs can be detected, as analysed by *in vivo* xenotransplantation into immunodeficient mice; also, haemopoietic progenitors are found at these early stages. Phenotypic characterization shows that HSCs and progenitors are in both the CD34-positive and CD34-negative fractions at week 6 of gestation and are exclusively in the CD34-positive fraction by week 19. These cells are in close association with the placental vasculature. The placenta may be considered a source of haemopoietic progenitors and HSCs in addition to

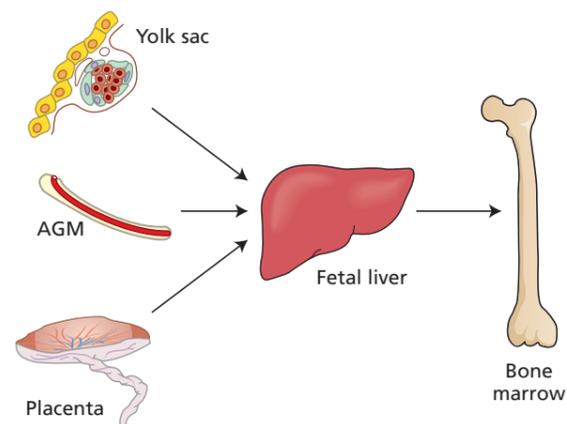


Figure 1.6 Haemopoietic sites during development. The first haemopoietic stem cells arise in the AGM region. Other haemopoietic cells and progenitors are generated in the yolk sac and placenta. It is as yet undetermined whether the yolk sac and placenta can generate haemopoietic stem cells. Haemopoietic cells generated in these three tissues migrate and colonize the fetal liver. Subsequently, the long-lived haemopoietic cells (primarily the haemopoietic stem cells) migrate and colonize the bone marrow, where they reside in the adult stages of life.

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umbilical cord blood for preclinical studies and potential clinical therapies.

HSC quiescence, proliferation and ageing

Somatic stem cells undergo lifelong self-renewal and possess the potential to produce the differentiated cells of the tissue. HSCs are considered to be relatively dormant stem cells, dividing rather infrequently. They are enriched in the quiescent fraction of adult bone marrow and are resistant to 5-fluorouracil (which is an antimetabolite drug that results in the death of rapidly dividing cells). Recent studies in mice using a label-retaining method for analysis of cycling versus non-cycling cells show that under homeostatic conditions, dormant HSCs cycle only once every 21 weeks. The adult mouse possesses approximately 600 dormant LSK CD150⁺CD48⁻CD34⁻ HSCs. Interestingly, 38% of HSCs in G₀, considered to be the dormant HSCs, can be activated by myelo/lymphodepletion during injury, 5-fluorouracil or G-CSF administration, and can return to the dormant state after the re-establishment of homeostasis.

The maintenance of HSC dormancy is thought to be an important strategy for preventing stem cell exhaustion during adult life. Serial transplantations in the mouse demonstrate that HSC self-renewal is limited to about six rounds of transplantation and that there is a progressive decrease in the ability of the transplanted stem cells to repopulate/self-renew. It has been proposed that accumulating DNA mutations and loss of telomere repeats adversely affect HSC function. Studies of chromosome shortening in human HSCs suggest that self-replication is limited to about 50 cell divisions. Recently, it was found that HSC characteristics are changed in aged mice. Comparison of various inbred mouse strains has shown that the rate of haemopoietic cell cycling is inversely correlated with their mean lifespan. The decrease in HSC quality was due to cell-intrinsic genetic or epigenetic factors. Causative genes were identified by transcriptional profiling comparisons between the HSCs of the different strains. Of particular interest are chromatin modifiers involved in prevention of HSC exhaustion through maintenance of a stem-cell-specific transcriptional programme. Changes in chromatin structure associated with high HSC turnover would result in stem cell senescence (which is thought to protect stem cells from malignant transformation by oncogenic events).

Transplantations of single HSCs from both the fetal liver and adult bone marrow have revealed HSC heterogeneity in lineage differentiation output related to developmental stage and aging: some HSCs give a balanced lineage differentiation output of myeloid and lymphoid cells, whereas others yield a predominant lymphoid or myeloid cell lineage output. During fetal stages, HSCs with a balanced lineage output are at a higher frequency than in adult BM. During aging the frequency of BM HSCs with a predominant myeloid output increases as compared to the frequency of HSCs with a balanced lineage output or predominant

lymphoid output. HSCs with a predominant myeloid output can also be found in prenatal life. Thus, the myeloid type HSC is not unique to aging – it is the prevalence to maintain these HSCs that is.

It is unclear why such heterogeneity in HSCs exists. HSCs generally do not undergo apoptosis in response to DNA damage and have adopted several mechanisms to preserve stemness rather than self-renewal, to reduce DNA damage and/or to prevent inappropriate differentiation leading to loss of HSCs. Both developmental and stem cell protective mechanisms may assist in providing maximum HSC fitness during reproductive life, providing an evolutionary benefit. Altered gene expression, however, may drive lymphoid differentiation, deplete lymphoid-biased HSCs and thus contribute to the relative predominance of myeloid-biased HSCs.

Haemopoietic-supportive microenvironments

Adult bone marrow microenvironment

Most tissue-specific stem cells are maintained in special microenvironments/niches that support long-term cell growth and self-renewal. To provide the continuous production of human blood over the many decades of adulthood, HSCs are maintained in the specialized haemopoietic-supportive niches of the adult bone marrow (Figure 1.2). The importance of the bone marrow haemopoietic niche and the interactions between supportive cells and HSCs was first demonstrated in mice. In transplantation studies of anaemic mouse strains naturally deficient in the c-kit receptor tyrosine kinase (W mice) or kit-ligand (KL; Steel mice) it was revealed that bone marrow from W mutant mice could not repopulate the haemopoietic system of wild-type irradiated recipient mice, whereas bone marrow from Steel mutant mice could. In contrast, W mutant mice could be repopulated by wild-type donor bone marrow cells, whereas Steel recipients were defective for repopulation by wild-type donor cells. It was proposed that a receptor–ligand interaction was involved to support HSCs within the bone marrow microenvironment. It was subsequently shown that HSCs express c-kit and bone marrow stromal cells express KL. The development of *ex vivo* culture systems to study this complex microenvironment allowed further dissection of the cellular and molecular aspects of the bone marrow microenvironment. These studies were aided by the isolation of mesenchymal stromal cells.

Stromal cell lines have been derived from the adult mouse bone marrow and fetal tissues. These are generally of mesenchymal lineage, as determined by cell-surface marker expression and their osteogenic and adipogenic potentials. Although widely heterogeneous in their ability to support haemopoiesis, some stromal lines (MS5 and AFT024, for example) have been shown

to support the growth and/or maintenance of HSCs in cocultures for long periods. Moreover, they have been instrumental in further characterization of these haemopoietic-supportive niches. Comparative transcriptional profiling and database analysis of HSC supportive and non-supportive stromal cell lines has revealed a complex genetic programme involving a wide variety of known molecules and molecules whose function in haemopoiesis is currently under investigation.

The *in vivo* bone marrow microenvironment is very complex, containing osteoblastic niches and vascular niches localized within the trabecular regions of the long bones. HSCs are maintained in close association with the so-called 'stromal cells' of the niches (osteoblasts and vascular endothelial cells). Along with KL, some of the key molecular regulators within the bone marrow niches include N-cadherin and CD150, and signalling pathway molecules SDF1, Notch, Wnt, Hedgehog, Tie2/angiopoietin, transforming growth factor (TGF), bone morphogenetic protein (BMP) and fibroblast growth factor (FGF). These regulators are implicated in a variety of cellular processes, such as HSC maintenance, differentiation, self-renewal and homing. Indeed, live tracking of haemopoietic progenitor/stem cells in the mouse model has shown the homing ability of these cells to bone marrow niches, and mouse models as well as *in vitro* culture systems are beginning to reveal the specific molecular mechanisms involved.

Microenvironments important for haemopoietic development in the conceptus

Prior to the establishment of an adult haemopoietic-supportive microenvironment, the embryo contains several haemopoietic microenvironments that are supportive and/or inductive. The extraembryonic yolk sac and placenta, and the intraembryonic AGM generate haemopoietic progenitor cells, whereas the AGM region generates the first adult repopulating HSCs (Figure 1.6). Little is known about the differences between the microenvironments of the embryonic haemopoietic tissues. However, the AGM microenvironment is the most-well characterized due to the simplicity of its structure, with the aorta at the midline of the embryo and the laterally located gonads and mesonephroi (Figure 1.5). The avian AGM microenvironment contains different types of mesenchymal cells and a population of aorta-associated stem cells called 'mesoangioblasts' that contribute to cartilage, bone and muscle tissues, and also to blood. In the mouse AGM region, cells more typical of mesenchymal stromal cells have been found. Interestingly, mapping and frequency analysis in the mouse conceptus show that mesenchymal progenitors, with the potential to differentiate into cells of the osteogenic, adipogenic and/or chondrogenic lineages, reside in most of the sites harbouring haemopoietic cells, suggesting that both the HSC and mesenchymal stromal cell microenvironment develop in parallel. Phenotypic characterization of haemopoietic-supportive AGM stromal lines places them in the vascular smooth muscle

cell (VSMC) hierarchy, in between a mesenchymal stem cell and a VSMC. Other niche cells include cells of the nervous system and endothelial cells.

Stromal cell lines established from the AGM region, placenta and fetal liver can support immature haemopoietic progenitors and HSCs and are more supportive as compared to adult bone marrow cell lines. Some can also support the haemopoietic differentiation of embryonic stem (ES) cells. Such stromal cell lines in a re-aggregate culture system have been able to support the differentiation of cells with a haemogenic endothelial phenotype (VE-Cad⁺CD45⁻CD41⁺ cells from mouse embryos before the onset of HSC generation) into long-term repopulating HSCs. This highlights that in an *ex vivo* controlled environment, cells with a potential to become HSCs, can be influenced to do so by other cells. However, it is still unknown whether the inductive factors in the stromal/re-aggregate cultures are the same factors produced in the *in vivo* physiologic HSC-inductive microenvironment. It is likely that HSC induction is a complex process requiring a variety of spatial and temporal cues emanating from several cell types in the niches of the embryo.

Within the normal physiology of the embryo, the AGM lies between the ventral tissue that includes mesenchyme and the endoderm-derived gut, and the dorsal tissue including the notochord and the ectoderm-derived neural tube (Figure 1.5). Mouse AGM explant culture experiments have shown that dorsal tissues/signals repress AGM HSC activity and ventral tissues/signals enhance HSC emergence. In both mouse and human AGM regions, cells expressing HSC markers are closely adherent to the vascular endothelium on the ventral aspect of the aorta. In the mouse, at precisely E10.5, single endothelial cells bud into the lumen as they take on HSC identity (Figure 1.5). Importantly, HSC activity, as determined by functional transplantation assays, is localized exclusively to the ventral aspect of the mouse mid-gestation aorta. Thus there is a strong positive ventral positional influence on HSC generation in the AGM, and morphogens and local signals emanating from the ventral endodermal tissues may be responsible for establishing the HSC-inductive microenvironment.

Haemopoietic transcription factors required for HSC generation such as *Gata2* and *Runx1* are expressed in cells of the ventral aortic clusters and endothelium. Deletion of *Gata2* and *Runx1* genes in mice leads to mid-gestation embryonic lethality, with complete absence of adult haemopoiesis (although embryonic haemopoiesis occurs), thus demonstrating that these two pivotal transcription factors promote the HSC genetic programme. Zebrafish and frog embryos have been useful models for dissecting the cascade of upstream events that lead to HSC induction. Developmental growth factor signalling pathways, such as the BMP, Hedgehog and Notch pathways, converge to activate expression of the two transcription factors in aortic haemopoietic cells and promote the HSC programme. In both the mouse and human embryo, BMP4 is expressed in the mesenchyme underlying the ventral aspect of the aorta at the time

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of haemopoietic cluster formation. Culture experiments have demonstrated the positive influence of BMP4 exposure to mouse and human HSC-containing cell populations. BMP4 has been found to act directly on HSCs in the AGM and, in addition, may stimulate the microenvironment to produce HSC effectors. Similarly, Hedgehog signalling regulates HSCs in the AGM region, likely in an indirect way through VEGF. Other ventrally localized HSC regulators include the Notch signalling molecules, as well as Wnt3a and interleukin (IL)-1.

High-throughput chemical screens offer a means of identifying molecules involved in HSC growth, maintenance and expansion. Through such a screen in zebrafish embryos, prostaglandin E2 (PGE2) was recently identified as a regulator of HSC number. When tested in the murine transplantation model, *ex vivo* exposure of bone marrow cells to PGE2 enhanced short-term repopulation by haemopoietic progenitors and increased the frequency of long-term repopulating bone marrow HSCs. PGE2 modifies the Wnt signalling pathway, which in turn is thought to control HSC self-renewal and bone marrow repopulation. Extracellular environmental cues, such as blood flow, also affect HSC generation. A zebrafish chemical screen identified modulators of blood flow such as nitric oxide synthetase (NOS). Inhibition or deficiency of NOS reduces murine bone marrow HSC number/function. Thus, together with general physiological cues, such as the haemopoietic growth factors, KL, IL-3, Flt3 and thrombopoietin, chemical modulators and developmental regulators may be useful for expansion of HSC number and enhancement of HSC function for therapeutic purposes.

Haemopoietic regenerative and replacement therapies

Stem cell transplantation

For over 50 years, HSC transplantation has been the most successful and significant clinical cell regenerative therapy (see Chapter 35). Initially, whole bone marrow was the source of cells used in clinical transplantation, but through experience and much research new and/or improved sources of transplantable HSCs were found. These now include the CD34⁺CD38⁻ fraction of adult bone marrow, mobilized peripheral blood HSCs and the CD34⁺CD38⁻ fraction of umbilical cord blood. The cumulative data from the large number of patients worldwide receiving a bone marrow transplant provide valuable information on the success of autologous versus allogeneic transplantation, the number of human leucocyte antigen (HLA) differences that are tolerated by the recipient, the incidence of graft-versus-host disease (GVHD), and the unexpected and advantageous graft-versus-leukaemia effect.

Interestingly, umbilical cord blood (UCB) appears to offer a beneficial source of HSCs for several reasons: UCB HSCs are

young, being harvested at the neonatal stage of development, thus circumventing concerns about the ageing of HSCs; UCB transplantation induces less frequent and less severe GVHD, since UCB contains many fewer activated T cells than adult bone marrow; also, UCB HSCs are highly proliferative. However, only relatively small numbers of cells are harvested (approximately 10-fold lower than those in adult bone marrow) and this limits their use to paediatric patients, unless multiple UCB units are transplanted. Despite increases in the number of UCB units (400 000) stored in cord blood banks (>50) around the world (catalogued and recorded by EUROCORD and other coordinating efforts) and HLA donor-cell selection for rare haplotypes, the supply of HSCs is still limited.

Gene therapy and gene editing for haemopoietic disease

Monogenic disorders of the blood are the first targets of gene therapy approaches. To effect a cure for a haematologic disease in which a single gene or regulatory element is mutated, a viral vector containing a normal copy of the gene is used to introduce and express the gene in HSCs. Gene therapy for β -haemoglobinopathies, such as β -thalassaemia and sickle cell disease, were among the first proposed and tested in mouse models. Primary immunodeficiencies (PID) are also monogenic disorders and result in the absence of (parts of) the innate and adaptive immune system. Patients can be cured with allogeneic HLA matched (related) HSC transplantation. However, donor availability is limited. For patients without an allogeneic donor, gene therapy of their own bone marrow HSCs and subsequent autologous transplantation is the only option for curative treatment. Lentiviral vector infection offers an efficient mode of delivery of a functional copy of the mutated gene into the genome of the patient's own HSCs used for transplantation. In initial gene therapy trials of immunodeficient patients, lenti-viral vector insertions in the genome of some transplanted HSCs resulted in activation of oncogenes, the selective growth of these HSC clones and the onset of leukaemia. More recent trials have incorporated a safety feature in the lentivirus that reduces (but has not completely eliminated) the unwanted activation of oncogenes in the case of viral insertion. In 2010 a new gene therapy clinical trial was initiated for Wiscott–Aldrich (WAS) patients who suffer from thrombocytopenia, eczema, recurrent infections, autoimmune disorders and high susceptibility to develop tumours. To date, all patients are alive and show significant increase in platelets and T cells, although long-term follow-up is required. Similar results have been obtained for adenosine deaminase-deficient severe combined immunodeficiency (ADA SCID) where 40 patients have been treated since 2000 in Italy, the UK and the US without any reports of malignant occurrences. X-linked SCID and chronic granulomatous disease (CGD) patients have also undergone gene therapy treatment,

albeit with less success. The treatment for CGD was impaired since the earliest trials did not make use of myeloablation to enhance chimerism of the gene-manipulated graft. Gene therapy trials for β -thalassaemic patients are ongoing and encouraging, but for successful treatment, higher levels of gene expression and HSC chimerism will be needed.

Clinical trials with gene therapy are promising. However, lentiviral vector insertions that may result in malignant clone outgrowth remain a risk. New gene editing techniques offer new hope for gene correction directly within the gene of concern. Gene editing makes use of endonucleases (zinc finger nucleases, TALENs or CRISPR/Cas9) to target a specific genomic site and repair the mutated gene or insert a functional gene under the control of its own promoter. This method leaves no extra genetic modulation. At present this approach requires prolonged cell culture and a selection step for the corrected HSCs, and thus requires further research developments in HSC growth and expansion before it will be clinically useful. Gene therapy for genetic disorders of coagulation proteins is discussed in Chapter 38.

New sources of HSCs for transplantation

The ability to expand HSCs *ex vivo* is a theoretically practical and attractive means to obtain an accessible and limitless source of HSCs for transplantation therapies. Unfortunately, despite many years of research using different culture systems and combinations of haemopoietic growth factors and proliferation-stimulating agents, *ex vivo* expansion of HSCs has not been achieved. However, HSC developmental studies have begun to provide new insights into the processes directing the generation and growth of HSCs. If cells such as the haemogenic endothelial cells of the embryonic aorta are present in the adult vasculature or could be obtained from ES/iPS cells, they could provide a novel source of inducible HSC precursors, particularly if they can be sustained and expanded to large numbers in culture.

Embryonic stem cells and induced pluripotent stem cells

Pluripotent embryonic stem (ES) and induced pluripotent stem (iPS) cells have been used to generate differentiated cells in many tissue systems, including the haemopoietic system. Developmental studies revealing the temporally and spatially limited production of HSCs in the embryonic vasculature, the components of the specific microenvironment, and the knowledge of the molecular programme of endothelial to haemopoietic cell transition have yielded insight into how HSCs may be induced and/or expanded without undergoing differentiation in such cultures. Haemopoietic-directed differentiation of human iPS cells towards endothelial cells, haemogenic endothelial cells and HSCs would be a potentially attractive alternative to conven-

tional sources of HSCs. Furthermore, such a cell culture system would make possible the use of novel gene editing approaches for monogenetic disorders. These gene correction approaches could be used in combination with patient-derived iPS cells. Studies using mouse and human ES cells have optimized culture conditions to include temporally changing combinations of growth factors (ActivinA, BMP4, VEGF, etc.) and signalling pathway antagonists to control differentiation to the mesodermal, vascular and thereafter the haemopoietic lineage. The ES-cell-derived haemopoietic cells arise from haemangioblasts and/or primitive endothelial-like cells that express PECAM-1, FLK-1 (KDR) and VE-cadherin, and are thought to represent the types of precursors, progenitors and differentiated cells found normally in the yolk sac. These results have strengthened the idea that ES cell differentiation proceeds via a 'haemogenic endothelial' differentiation step, before definitive haemopoietic cells can be produced and require activation of the Wnt- β -catenin pathway (Figure 1.7).

An alternative approach to produce HSCs *ex vivo* has recently emerged. This molecular reprogramming approach aims to reprogramme non-haemopoietic or differentiated haemopoietic cells directly to HSCs, without going through a pluripotent stem cell state. Such induced HSCs, or iHSCs would be generated through reprogramming directed by transcription factors pivotal to HSC generation and/or growth. Several laboratories have been able to generate haemopoietic progenitors or stem cells from more differentiated cells using four to eight different haemopoietic transcription factors previously identified from HSC transcriptome databases. In one case, mouse endothelial-like precursor cells (Sca1+, Prominin 1+ and expressing a human CD34 reporter) have been converted into haemopoietic progenitors using the factors Gata2, Gfi1b, cFos and Etv6. A human myeloid precursor (CD34+CD45+) cell has been converted into a haemopoietic progenitor cell (CD34+CD38-) using HOXA9, SOX4, RORA, and MYB; however, neither study was able to generate long-term repopulating HSCs. A study converting mouse committed B cell progenitors using a mix of eight transcription factors (Run1t1, Hlf, Lmo2, Prdm5, Pbx1, Zfp37, Myc-n and Meis1) has resulted in long-term repopulating HSCs. In this method, B cell progenitors are transduced with the eight factors, and immediately transplanted into irradiated recipients. In this way the native bone marrow niche preserves the new iHSCs and allows them to be maintained and function in the physiologic context of the recipient (Figure 1.7). Whereas this study demonstrates that transcription factor transduction of haemopoietic cells can yield HSCs, this approach is limited in applications for research or therapy. The fact that each of these studies uses a completely different panel of transcription factors to make induced HSCs or HPCs indicates that there may be more than one way to reprogramme cells to the haemopoietic lineage and that a further understanding of HSC biology is required.

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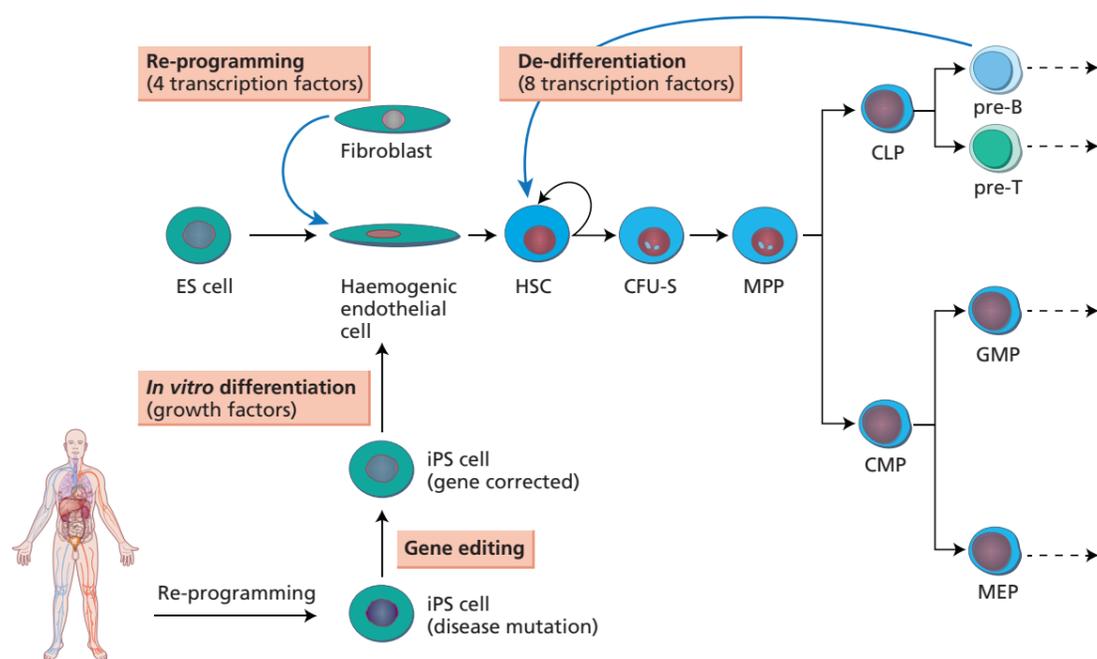


Figure 1.7 Several experimental approaches to generate HSCs *de novo*. De-differentiation of pre-B cells with eight transcription factors and immediate transplantation *in vivo*, allows for the production of multilineage, self-renewing HSCs due to the presence of functional HSC niches that are as yet not attainable in *in vitro* cultures. To date, direct reprogramming of B cell progenitors and immediate transplantation into irradiated mouse recipients has been the only study successful in generating HSCs. Reprogramming with four pivotal haemopoietic transcription factors has yielded haemogenic endothelial cells and haemopoietic

progenitors, but not HSCs. *In vitro* haemopoietic differentiation of ES and iPS cells relies on the addition of developmental and haemopoietic growth factors to induce the progressive differentiation of these pluripotent cells to mesoderm, endothelial, haemogenic endothelial and haemopoietic fates. As this culture system improves, it may be possible to make iPS cells from patients with monogenic disease and correct the gene mutation by gene editing. These cells may then be differentiated to HSC fate and used for clinical treatment.

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