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Identification of Unique
Hematopoietic Stem Cell Properties

by

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With the approval of the Lund University Faculty of Medicine, this thesis will be defended on November 25, 2005, at 9:00, in Segerfalksalen, BMC, Lund.

Faculty opponent:
Prof. Ihor Lemischka, Department of Molecular Biology,
Princeton University, Princeton, USA

Lund University
2005
Till Josefine och Max
On the cover
Infarcted left ventricular myocardium with spared endocardium (red) and donor derived (green) blood cells (white). Cell nuclei are in blue.
The thesis is based on the following papers, referred to in the text by their Roman numerals (I-III).


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<td>AGM</td>
<td>Aorta, gonads and mesonephros</td>
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<td>ALL</td>
<td>Acute lymphoid leukemia</td>
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<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>CFU-S</td>
<td>Colony forming unit spleen</td>
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<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
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<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
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<td>CMP</td>
<td>Common myeloid progenitor</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dpc</td>
<td>days post conception</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
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<td>GMP</td>
<td>Granulocyte macrophage progenitor</td>
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<td>HSC</td>
<td>Hematopoietic stem cell</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>lacZ</td>
<td>β-galactosidase gene</td>
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<td>LMPP</td>
<td>Lymphoid primed progenitor</td>
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<td>LT</td>
<td>Long-term</td>
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<td>MDS</td>
<td>Myelodysplastic syndromes</td>
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<td>MkEP</td>
<td>Megakaryocyte erythroid progenitor</td>
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<td>NK cell</td>
<td>Natural killer cell</td>
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<td>P-Sp</td>
<td>Para-aortic splanchnopleura</td>
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<td>PCV</td>
<td>Polycytemia Vera</td>
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<td>S/G2/M</td>
<td>Cell cycle phases Synthesis, Gap 2 and Mitosis</td>
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<tr>
<td>SCID</td>
<td>Severe combined immuno-deficiency</td>
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<td>ST</td>
<td>Short-term</td>
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HEMATOPOIESIS

Since the beginning of time, man has admired Nature. Through the ages he has illustrated, hypothesized and described it to somewhat come closer to understanding it. This is particularly true when it comes to science, where one of our strongest inspirations, to look for the unknown or explain the unexplained, is our respect and admiration toward what we explore. To me, perfection in Nature is when it acts in balance and synergy to create something bigger than itself. No matter if it is molecules interacting in a cell membrane, bacteria absorbing nitrogen in the roots of a plant or a wolf pack hunting a prey. When autonomous units in Nature work in synergy, with a common goal and purpose, they become something bigger than the sum of what they are as independent units.

In all vertebrates and some invertebrates hematopoietic cells act in synergy to create a defense against infections. Each of the different hematopoietic cell types has their own function and properties, but it is only when they act in tight collaboration that they constitute a highly specific and efficient immune defense that can fight infections from invading pathogens. All these hematopoietic cell types are continuously produced in the bone marrow (BM) by rare stem cells that persist throughout life of the organism, the hematopoietic stem cells (HSC).

THE HEMATOPOIETIC SYSTEM

The HSCs produce hundred of millions (~2.4x10^8) of new blood cells everyday to meet the demands of the immune system (1). To understand the purpose and necessity of normal blood cell production (hematopoiesis) from these stem cells it is important to know what cells they produce as well as their properties and
actions. Hematopoiesis is highly conserved through evolution and is largely similar between lower vertebrates and mammals (2, 3). The focus of this thesis is on mouse HSCs, but conceptually most aspects can be translated into humans as well.

**THE BLOOD CELLS**

Microorganisms (pathogens) such as viruses, bacteria, fungi and parasites, cause infectious diseases. In 1796 Edward Jenner discovered that cowpox, or vaccinia, through vaccination gave protection against human smallpox, a pioneering discovery that gave one of the first clues on how the immune system works and that paved the way toward eradication of smallpox in 1979. Today, we know that the primary elements behind this *adaptive immune response* are immunoglobulins that bind to the pathogen, and that they are produced by lymphocytes. Other cells and molecular components make out the *innate immune response* following infections, and have a more direct action against pathogens, mainly through phagocytosis and destruction (4).

The hematopoietic blood cells (Figure 1), originate in the BM from a common precursor, the HSC (1). Differentiation from these precursors to the mature blood cells is a tightly regulated multi-step process involving close interaction with other cells and soluble factors in the BM microenvironment and results in the production of various mature blood cell types (1). Among these are *platelets* and *erythrocytes*, that are produced by the millions each day by the megakaryocyte/erythrocyte progenitor (MkEP) and released to the circulation (5). Large megakaryocytes shed small enuclear platelets that circulate in the blood and participate in blood clotting. Erythrocytes are the most common blood cells and deliver oxygen from the lungs to the tissues via the blood. They lack nucleus and organelles and have a biconcave shape that allows optimal exchange of oxygen and makes them flexible to fit through tiny capillaries. Both platelets and erythrocytes are fairly short-lived and destroyed in the spleen.
Granulocytes and macrophages are involved in the innate immune response and their production, from the granulocyte/macrophage progenitor (GMP), can be exponentially increased in the BM to enable release of large number of cells that migrate to sites of infection or inflammation (5). The neutrophils, basophils and eosinophils are all granulocytic cells and have a short life-span following release into the circulation. Neutrophils are phagocytes and the most important component of the innate immune response, indispensable for the defense from bacterial infections. Basophils and eosinophils are mainly important in the defense against parasitic infections. Macrophages are the mature form of immature circulating monocytes that differentiate upon

Figure 1. A model for the hierarchical differentiation from HSCs into the specialized blood cell types through committed progenitor cells with limited self-renewing capacity.
migration into tissues and are an important component of the innate immune defense by fast clearance of pathogens and infected cells through phagocytosis. **Mast cells** are very similar to basophils but originate from a different precursor in the BM (6). The role of mast cells in the immune response is mainly in the protection of mucosal surfaces but they play also a role in the allergic response. The above cell types are all commonly referred to as **myeloid cells** and originate from common myeloid progenitors (CMP) or lymphoid primed progenitors (LMPP) in the BM (Figure 1).

**Lymphoid cells** originate from common lymphoid progenitors (CLP), that are the offspring of LMPPs in the BM (7), and consist of **Natural killer** (NK) **cells**, **B cells** and **T cells** (Figure 1). The NK cells are a component of the innate immune defense. B and T cells are effectors of the adaptive immune response and are small with few organelles and condensed nuclear chromatin, typical of inactive cells. They have no functional activity until they encounter specific antigens that bind to highly specific receptors on their cell membrane and stimulate proliferation and differentiation (4). The receptors on each cell recognize only one type of antigen, but together the total receptor repertoire of all cells recognizes a wide diversity of antigens. **B cells** mature in the BM and bear receptors on their membranes that have the same specificity as the antibodies they will release when they, upon activation, differentiate into antibody producing plasma cells (4). **T cells** are generated in the thymus but their progenitors have a BM origin and migrate to the thymic microenvironment (8, 9) for maturation into the two main types of T cells (cytotoxic and helper T cells) (4). These both bear receptors that are highly specific for a variety of antigens. Foreign protein fragments, presented on major histocompatibility complex receptors, on pathogen-infected cells are recognized by receptors on cytotoxic T cells and leads to destruction of the infected cell. The helper T cells regulate the activity of other hematopoietic cells like B cells and macrophages. When the B cells and T cells have matured in the BM and thymus respectively,
they migrate to the peripheral blood and the peripheral lymphoid organs (lymph nodes, spleen and mucosal lymphoid tissues) where phagocytic cells carrying antigens from sites of infection interfere with receptors on B and T cells to initiate the adaptive immune response.

THE IMMUNE RESPONSE

The action of these immune effector cells following an infection results in an immune response with the purpose of eliminating the pathogen, as is described very generalized below. Upon infection, phagocytes and other components of the innate immune system provide a first line of defense. These cells have receptors that recognize mostly bacterial surface antigens triggering phagocytosis or secretion of factors that lead to responses commonly known as inflammation. As the diversity of these receptors is low and limits the specificity of the innate immune response, a complementary system involving the lymphoid cells has evolved to enable fast and versatile adaptation to changes in pathogen recognition (4).

The cytotoxic T cells eliminate cells in the organism that have been infected by pathogens (mostly viruses). The foreign proteins produced in the infected cell are presented on the cell membrane and as they are recognized as non-self antigens by the receptors on the cytotoxic T cells, this leads to the killing of that particular cell. Upon binding of an antigen to the immunoglobulin receptors on a B cell and activation by interaction with a helper T cell, the B cell starts to proliferate to generate a clone of cells with identical antigen specificity. This is followed by differentiation into plasma cells that secrete antibodies that are the soluble forms of their immunoglobulin receptors. The binding of antibodies to antigens leads to activation of the innate immune response, resulting in neutralization, opsonization or plasma protein (complement) activation and ingestion of the pathogen by phagocytic cells. As remnants of the clones of activated B cells are preserved for a long time after an adaptive
immune response, subsequent infections from the same type of pathogen result in a much more efficient activation of the immune response and therefore also prevents the pathogen from establishing and expanding to generate a disease (4). It was this mechanism that Edward Jenner discovered and that is now widely used to provide protection against pathogen infections by vaccination.

**DEVELOPMENT OF HEMATOPOIESIS**

The onset of production of the broad repertoire of hematopoietic cells is an early event in embryonic development to meet the demands of oxygen transportation as the embryo becomes larger and to provide an early defense against pathogens. For this, an exponential increase in the production of blood cells from HSCs is required.

**EMBRYONIC HEMATOPOIESIS**

In mouse development, gastrulation starts 7.5 days post conception (dpc) and leads to the formation of ectoderm, mesoderm and endoderm. During this process the extra- and intra-embryonic regions, the yolk sac and embryo proper, are established. In the yolk sac, cell aggregates called blood islands are formed and contain cells of both hematopoietic and endothelial lineages (Figure 2). These develop in close contact, and perhaps from a common progenitor cell known as the hemangioblast (10-12). Commitment during development toward a hematopoietic fate occurs through the influence of various transcription factors, such as Tal-1/SCL, AML-1, Lmo2 and GATA-2, and results in the formation of committed **primitive hematopoietic precursor cells** (13). In the yolk sac such primitive precursors have a myelo-erythroid potential and mostly produce monocytes, for infectious defense in the placenta, and primitive erythrocytes, that are large, nucleated and produce embryonic globins (14). This early burst of
extra-embryonic erythrocyte production is necessary for oxygen transport within the embryo as it grows bigger, when oxygen diffusion from maternal circulation becomes insufficient (15). The yolk sac remains a hematopoietic organ until the embryo itself can support blood cell production by around 11.5 dpc.

Within the embryo, a region comprising the rudiments of the dorsal aorta and surrounding splanchnic mesoderm forms at around 8.5-10 dpc and in this region the development of definitive HSCs initiates (16-18). This area, named the para-aortic splanchnopleura (P-Sp), later develops into the aorta, gonads and mesonephros (AGM) at 10-12 dpc (Figure 2). Hematopoietic precursor cell activity can be identified by 10.5 dpc in a region of the mesenchyme surrounding and within the dorsal side of the aorta (16-18). These precursors support definitive hematopoiesis, produce small, enucleated erythrocytes expressing adult globins and develop into the definitive HSCs. As these HSCs mature, they migrate and enter the blood circulation (19, 20) to colonize the liver (21) (Figure 2). In the fetal liver environment, the HSCs proliferate and self-renew rapidly to expand their numbers and to meet the growing requirements of the hematopoietic system (22-24). This is reflected by a long-term repopulation potential that exceeds that of adult HSCs (24-26). The fetal liver (12-14 dpc) hematopoiesis alleviates the necessity of yolk sac

Figure 2. Spatial and temporal development of primitive and definitive hematopoiesis in the mouse embryo.
erythropoiesis, and the yolk sac hematopoietic precursors thereafter undergo programmed cell death and disappear. Thus, erythrocyte globin switching, from fetal to adult, might be due to changes in cell populations rather than transcription. By the end of pregnancy, hematopoiesis in the liver transfers through the migration of HSCs to the BM (19, 20, 27), which remains the main hematopoietic organ throughout life (1) (Figure 2). Both HSCs and mesenchymal progenitor cells (with osteogenic, adipogenic and chondrogenic potential), that are present in most of the sites harboring HSCs throughout ontogeny, home to and develop niches supporting self-renewal in the primary hematopoietic organs (fetal liver, BM and spleen) as these tissues develop in the fetus (19, 27-29). Whether such colonization of niches supporting hematopoiesis during mid-gestation is a multi-wave process or the result of a constant flow of rare HSCs in the fetal blood is under debate (19, 20, 27). Probably low numbers of HSCs are constantly circulating both before and after their expansion and maturation in the fetal liver.

ORIGIN OF DEFINITIVE HEMATOPOIESIS

Both embryonal and adult hematopoiesis is hierarchical and differentiation occurs through distinct progenitor subsets (30). This suggests that the molecular mechanisms underlying cell fate decisions are conserved from embryo to adult. Erythropoiesis and necessary but limited blood cell production in the embryo can be provided by both primitive hematopoietic precursors in the blood islands of the yolk sac and definitive HSCs forming in the P-Sp/AGM region. Nevertheless, intra-embryonic definitive HSCs are the sole precursors of the adult HSCs that supply HSC activity throughout life (15), and no further HSCs are generated during late fetal and neonatal stages of development (31).

The origin of intra-embryonic definitive HSCs has been under debate as to whether they are descendants from the primitive yolk sac derived precursors, dependent on primitive hematopoietic cells for proper maturation within the
embryo or originate independently from definitive hematopoietic precursors. Studies by Moore and Metcalf in 1970 (32) suggested that the yolk sac is required for both primitive and definitive hematopoiesis in mice. Culture of pre-circulation 7 dpc embryos from which the yolk sac had been removed developed without blood cell formation, whereas 7 dpc yolk sac alone yielded abundant hematopoietic colonies. These findings have been challenged by more recent and similar studies suggesting that definitive HSCs are only of intra-embryonic origin and that the yolk sac cells can only provide short-term myelopoiesis (33, 34). A third option, that requires advanced lineage tracing experiments to address (31), could be that extra- and intra-embryonic hematopoietic cells share the same precursor in the yolk sac and P-Sp/AGM region of the developing embryo. Their distinct developmental fates would then be determined by extrinsic environmental cues that are different in extra- and intra-embryonic niches (35-38). In support of this, transplantations of Xenopus yolk sac-like region to the AGM-like region causes these cells to adopt a definitive fate (39).

HEMATOPOIETIC STEM CELLS

In the early 1960s, McCulloch and Till performed a series of ground breaking experiments to investigate the existence of stem cells in the BM with multipotent and self-renewing capacity. The experiments involved injection of BM cells into irradiated mice and subsequent evaluation of visible nodules from the transplanted cells in the spleens of the mice. Nodules appeared in proportion to the number of BM cells injected and arose from single BM cells (40), named colony forming units spleen (CFU-S). That these CFU-S were able to self-renew (41), which is a crucial aspect of the functional definition of stem cells, was the first true evidence for the existence of an adult HSC in the BM. The following
chapter is a conceptual, rather than detailed, introduction into HSCs, their identity, regulation and fate.

DEFINITIONS
At the end of fetal maturation, the BM is the primary hematopoietic organ. The seeding of HSCs to the BM during late gestation is a uniform process, without regional patterning, that results in a homogeneous distribution to the different BM compartments without spatial differences in hematopoiesis or HSC identities (42). The majority of cells in the BM are maturing blood cells and their progenitors, and the HSCs therefore constitute a rare population of less than one in 15,000 BM cells (43). The identity of these rare BM HSCs cannot be recognized by their morphology and phenotype alone, but only by unique functional properties that distinguish them from all other cells in the hematopoietic organs (44-46).

Adult HSCs are largely quiescent, in that their transit through the cell cycle is slow or even arrested at times. This is reflected by as few as 8% of the HSCs entering cell cycle each day, but nonetheless within 4-8 weeks most have divided at least once (47, 48). The cell cycle is the series of events in a eukaryotic cell from one cell division to the next (Figure 3). It consists of distinct phases (49) in which the cell undertakes sequential actions like growth and preparation of the chromosomes for replication (G1 phase), DNA synthesis to duplicate the chromosomes (S phase), additional growth and preparation for cell division (G2 phase) and finally, mitosis (M phase) which is the actual division of the cell into two daughter cells. The cell cycle is regulated at checkpoints (49-51) during each phase-transition by cyclins that form complexes with cyclin-dependent kinases (49, 52). This cyclin-based surveillance system acts as a quality control that monitors the cell as it progresses through the cell cycle. Checkpoints can block progression through one phase if certain conditions are not met. For instance, mitosis is inhibited
until DNA replication is completed or if not all chromosomes are attached to the mitotic spindle. If this system senses a problem, a network of signaling molecules instructs the cell to stop dividing to either repair the damage or initiate programmed cell death. This ensures that damaged cells are not further propagated and do not progress into a cancerous state.

Some cells leave the cell cycle at the G₁ phase following a cell division and enter a quiescent G₀ stage (51). Often G₀ cells are terminally differentiated and have therefore permanently exited the cell cycle whereas other cells, like the HSCs, are only temporally quiescent and can upon mitotic stimulation re-enter G₁ and prepare for additional cell cycles (47). HSCs take a longer time than committed progenitor cells to respond to growth factor stimulation, as they first

**Figure 3.** The HSC cell cycle with distinct G₀ and G₁ phases typical of quiescent cells. Indicated are three major cell cycle checkpoints. At the G₁ checkpoint it is controlled whether the cell is big enough and the environment suitable to proceed. At the G₂ checkpoint it is verified that DNA replication has been completed and successful and at the M checkpoint that chromosomes are properly aligned and attached to the mitotic spindle.
need to get activated to re-enter the cell cycle. Thus, the relative unresponsiveness of HSCs to mitogenic stimuli (47, 53, 54) might reflect their predominant G₀ state. Distinct regulation of the cell cycle activity of HSCs by factors known to limit proliferation and differentiation (55-60) could be a requirement to avoid exhaustion of the HSC compartment (61) and represent a defining stem cell property. Active cell cycling have been suggested to exert negative effects on stem cell function (62-66). This can at least in part be an interpretation of experimental observations as the design of such experiments have assumed both that dividing hematopoietic stem and progenitor cells have a similar cell cycle transit time and that HSCs are identifiable by phenotype alone.

As HSCs divide, they produce daughter cells that are identical replicas. Such self-renewing cell divisions are a hallmark of stem cells and necessary to maintain a constant HSCs pool and lifelong production of all blood cell types (41). Maintenance of the HSC pool can be the result of either asymmetrical cell divisions (67) that results in one cell that is identical to the mother cell and one cell that is committed to differentiation. Similar maintenance could also be the result of a balance of symmetrical cell divisions leading to either complete self-renewal or differentiation (i.e. result in either two HSCs or two committed daughter cells). Nevertheless, HSC asymmetric cell divisions must occur at some point during development and multilineage differentiation of committed cells (68). Cell intrinsic alterations, through yet undiscovered mechanisms, occur as the HSC age (69, 70) and DNA damage accumulates over time due to their high number of cell divisions. This is limited by tight control over self-renewal and proliferation by several regulatory pathways, such as the Bmi-1 (59), Wnt (71), Gfi-1 (57) and c-Myc (72) pathways, and DNA integrity is maintained through cell cycle checkpoint surveillance (55) and the exclusive preservation of the chromosome ends (telomeres) by the reverse transcriptase telomerase (73-75).
HSCs are multipotent and the sole precursor in adults that can produce all the different types of hematopoietic blood cells. The mechanisms that underlie and direct the multilineage commitment processes are still largely unknown but a descriptive model (Figure 1) of the differentiation process has been established (43, 76). In this, self-renewing and multipotent long-term HSCs (LT-HSC) exist throughout life (46). Their committed progeny irreversibly transit into short-term HSCs (ST-HSC) that are also multipotent but contribute to hematopoiesis for a shorter time (less than 6-8 weeks) as they are no longer capable of extensive self-renewal (43, 77). The ST-HSCs next commit and becomes progenitors with more restricted lineage potentials and these in hand develop along certain cell lineage pathways with sequential restrictions in lineage potential and gene expression as a consequence (5, 7, 76).

Under physiological conditions, but more pronounced during stress, HSCs migrate in and out of the BM compartment. This occurs at a very low frequency (78-80) by a mechanism that can be stimulated by exogenous cytokine treatment (81). The purpose and regulation of this migratory activity is not completely understood but might play a role in the seeding of HSCs to other niches within the BM (79, 80) or other organs (82). Efficient homing from the liver to the BM during fetal development (20) and to the BM following therapeutic or experimental transplantation (45) has been suggested to be a unique property of HSCs.

Taken together, quiescence, lifelong maintenance of HSCs and multilineage blood cell production are unique properties possessed by HSCs alone.

REGULATION
In light of the relatively short life span of a mouse (~2-3 years depending on strain) the low cell cycle activity among HSCs, with a population turnover of up to six months (48), is remarkable. If the steady state is disrupted, for example by pathogen infection or hemorrhage, HSCs are rapidly activated to meet the
demands on increased blood cell production. Similarly, following manipulative treatments such as myeloablation and BM transplantation, HSCs react by rapid expansion in the recipient (61, 75, 83) and under the influence of exogenous growth factors, commitment, migration or even self-renewal might occur (81, 84, 85). Taken together, this argues that the activity of the HSC needs to be tightly regulated to fulfill the requirements of the organism in different physiological conditions. It is currently unknown which state is the default fate (if any) of HSCs but most likely it is to commit and differentiate (Figure 4). Thus all other fate decisions, including to remain uncommitted, need to be influenced by various extrinsic and intrinsic regulatory factors. Identification of these factors and their signaling pathways has been one of the main focuses of hematological research, as knowledge on how HSCs are regulated would allow manipulation for therapeutic purposes.

Extrinsic factors are produced either by the stem cells themselves or by surrounding stromal cells and bind to molecules (receptors) on the cell membrane of the stem cell (Figure 5). Their effect can be long range, as soluble molecules, or local, through direct cell-to-cell contact between stem cells and adjacent cells (86). The stromal cells and their products are spatially distributed
into niches that differ in their HSC maintenance capacity and therefore homing of HSCs and hematopoietic progenitor cells to different niches affects their fate and regulation of hematopoiesis (87-89).

The best evidence for such stem cell niches has been found in the Drosophila testis where germline stem cells surround apical hub cells at the tip of the testis, which provide self-renewing signals (90, 91). As the stem cells divide, the daughter cell that keeps contact with the hub cells, and thereby continues to receive self-renewing signals, retains a stem cell identity. The other daughter cell that is relocated away from the hub cells initiates differentiation. In the BM, similar regional patterning of self-renewing signals has been found within the endosteal zone lining the bone surface in the marrow cavity (87-89), with a gradient decreasing toward the central zone of the marrow space. In support of this, the majority of hematopoietic stem and progenitor cells has long since been known to be distributed preferentially along the bone surface (92-94). These findings suggest that fate determination of HSCs within the endosteal zone occurs in a similar fashion as for germline stem cells in Drosophila testes, where the fate of the two daughter cells from a dividing HSC is determined by their attachment to or displacement from the stem cell-supportive cells that make up the stem cell niche.
The asymmetry of the two daughter cells derived from HSC self-renewing divisions might also be due to asymmetric distribution of intrinsic factors (Figure 5), such as transcription factors, during cell division (68, 95). Most likely, HSC regulation is a complex process involving both intrinsic and extrinsic factors that can be both counteracting and synergistic. For example, asymmetric cell division might be dependent on extrinsic signals that prime HSCs for subsequent intrinsic regulation and might explain why extensive efforts to \textit{ex vivo} expand HSCs so far has, with some exceptions, been fruitless (96-102).

FATE DECISIONS

Following a HSC division, the interaction of the two daughter cells with their environment results in fate decisions that determine the destiny of each particular cell and such interaction continues throughout the life of the cell (Figure 6). The newly formed cells can either return to a \textit{quiescent state} as the parental HSC, carry on a second \textit{self-renewing} asymmetric (or symmetric expanding) cell division, \textit{commit to differentiate} along a certain lineage pathway or \textit{migrate} to a distant site that offers suitable environment for either of the fates above (103). If the environment does not support any of these possibilities, a last option is to undergo \textit{programmed cell death} (apoptosis).
The regulation of HSC fate decisions (Figure 7) is most likely a combination of stochastic (random) events, mainly though intrinsic regulation at the time of cell division (95, 104-107), and deterministic events, mainly due to extrinsic factors in the HSC niche, that are either permissive or instructive in their action (108-110). Eliminating single or multiple hematopoietic growth factors or signal transduction pathways, by genetic engineering in mice, allows determination of the type of action a factor imposes on the HSC. The action of some factors is redundant as their removal does not result in hematopoietic phenotypes and can be compensated for (111-113) whereas others are indispensable for certain fates. The first case exemplifies permissive regulation, allowing cells to differentiate along a predestined differentiation pathway, and the latter instructive regulation, instructing cells toward a specific fate (114).

A group of extrinsic factors, called cytokines, play an important role in regulation of hematopoiesis. These molecules are available both in soluble and cell membrane bound forms and interact by direct binding to cell membrane receptors on the hematopoietic cells or intermediate cells with which the

![Figure 7. Regulation of HSC fate decisions by intrinsic stochastic events and deterministic events due to extrinsic factors.](image)
hematopoietic cells interact. The early acting cytokines stem cell factor and thrombopoietin are non-redundant regulators of the HCS-pool (13). Other cytokines act on more committed cells and drive differentiation along particular pathways, like erythropoietin for erythroid cells, thrombopoietin for megakaryocytic cells, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) for myeloid cells and interleukin 7 (IL-7) for B cells (13, 115).

Signaling from membrane bound receptors on HSCs is propagated through elaborate intracellular signal transduction pathways to the nucleus, where they influence the activity of transcription factors. These bind to promoter elements on the DNA and regulate together with other regulatory molecules and DNA polymerases the transcription of target genes and ultimately, fate decisions. Several transcription factors have been implicated in the regulation of HSC self-renewal (ICN/CSL, Ikaros, HoxB4 and GATA-2), whereas others participate in commitment toward a myeloid (PU.1 and GATA-1) or a lymphoid (PU.1, Ikaros, GATA-3, ICN/CSL and E2A) fate (for review see reference 13).
Transplantation of HSCs has developed from an experimental therapy for a small group of patients to a well-established form of treatment for a large group of patients with hematological and non-hematological disorders. Cellular and genetic engineering of HSCs prior to transplantation would allow for additional potential applications of such transplantations.

LEUKEMIAS
Leukemias have long since been classified based on the morphological characteristics that are the consequence of chromosomal abnormalities (mutations) and their resultant malignant differentiation (116). The sub-classes are heterogeneous, comprising diverse types of abnormalities with different origins, properties and prognoses, but serve as a useful tool for efficient diagnosis and treatment. Treatment of patients with leukemia and lymphoma has developed significantly over the last few decades by improved classification (diagnosis), prognostic risk stratification, relief of symptoms and eradication of the malignant cells.

Acute myeloid leukemia (AML) is the most common type of adult leukemia and typically involves a differentiation block of myelopoiesis. The accumulation of undifferentiated myeloblasts in the bloodstream and vital organs results in symptoms such as anemia and abnormal bruising. AML can be preceded by the chronic myelodysplastic syndromes (MDS), polycytemia vera (PCV) or chronic myeloid leukemia (CML) and have in such cases a worse prognosis. The myeloid differentiation defect in AML is most likely not enough to develop the leukemia. Rather, other effects from the translocations are most likely decisive such as, increased self-renewal, reduced DNA-repair and reduced
apoptosis. Treatment is usually chemotherapy, but for high-risk patients, allogeneic BM transplantation (see next section) is the best option.

**Acute lymphoid leukemia** (ALL) has a lymphoid phenotype and is the most common leukemia in children, for which the cure rate is high (except in infants). From studies of twins it is known that the leukemic translocation can occur during pregnancy (117) but as such aberrations can also be present in healthy individuals they are most likely not sufficient for transformation (118). Treatment depends on the type of translocation but it is usually chemotherapy.

Cell fate decisions in leukemic cells are abnormal due to mutations in genes (oncogenes or tumor suppressor genes) that are indispensable for normal regulation of cell proliferation and differentiation. Such mutations usually lead to gain of self-renewal ability, increased proliferation capacity, blocked differentiation, avoidance of apoptosis and higher susceptibility to secondary mutations (116). The leukemic initiating event occurs in a cell that becomes the ancestor of all other leukemic cells. Chromosomal abnormalities commonly found in patients with leukemia can also be found in healthy individuals and argues that some mutations are not transforming the cell but making it vulnerable to secondary leukemia-initiating mutations (119).

Some of the defining properties of stem cells are shared with leukemic cells, but as the regulation of these properties (self-renewal, apoptosis, drug resistance) in transformed cells is disrupted, they become malignant. Gain of self-renewal capacity is a requirement for infinite production of malignant cells and results in a **cancer stem cell** that is both required and sufficient for development and maintenance of the disease (118, 120-122). The identity of such cells in different leukemias have been investigated, and it has been established that the transformation event can occur both in HSCs and in committed progenitor cells (121-125). The lineage-restricted pattern of some leukemias suggests that the transformation either occurred in a progenitor cell with restricted lineage potential (120) or, in a HSC where the heterogeneity
depends on altered abilities to differentiate in the developing leukemia (120, 124, 126). The latter idea has been supported by recent studies suggesting that the initial transformation might occur at the stem cell level and that subsequent events affecting the malignant phenotype might occur in a committed progenitor that leads to the acute phase of the disease (127).

**BONE MARROW TRANSPLANTATION**

In treatment of leukemia, chemotherapy is sometimes not enough to completely eradicate the malignant cells and in such cases replacement of the patients malignant cells by BM transplantation might be an option. Almost 50 years ago, it was demonstrated that such BM transplantation could rescue lethally irradiated mice from the myeloablation effects (128). Since then, the technique has been refined into a therapeutic treatment for leukemia and that work, by Dr. E Donall Thomas, was awarded the Nobel Prize in 1990 (129). Transfusion of BM cells to patients with hematological disorders can be done with cells from human leukocyte antigen (HLA) matched donors (usually siblings, but also unrelated). Such allogeneic transplantation provides favorable graft-versus-leukemia effects but can also result in deleterious graft-versus-host disease. In some cases donor cells are taken from the patient before myelosuppressive treatment and re-infused to rescue normal hematopoietic function. Such autologous transplantations avoid graft-versus-host reactions, but carry the risk of leukemic relapse from rare residual leukemic cells.

**EX VIVO EXPANSION**

In some transplantation settings the low HSC numbers in the donor cell source prevents successful engraftment. An example is the use of HSC from the umbilical cord blood for allogeneic transplantations. The low numbers of HSC in each umbilical cord currently limits their use to children patients, but as the fetal HSCs have less graft compatibility problems in allogeneic transplantations,
extending their use to adults would be desired. *Ex vivo* manipulation of donor preparations to stimulate expansion of their HSC numbers would be a means to circumvent such problems, but so far has had limited success (96-102).

To expand limited numbers of HSCs in donor BM, umbilical cord blood or blood, most strategies have focused on single or multiple growth factor treatments. Cytokines that alone can not support HSC expansion, have been used in combination to maintain or expand HSCs numbers *ex vivo* (98, 99). Despite promising initial results in transgenic models, signaling pathways like the Notch (130), FGF (131), Wnt (71, 132) and HoxB4 (133-135) pathways have not yet been successfully used to establish an effective *ex vivo* expansion protocol.

In addition to being important for developing BM transplantation protocols, conditions that both maintain and expand the numbers of HSCs *ex vivo* will be crucial to developing gene therapy protocols. Such treatments may offer gene correction or protein expression rescue therapies of several BM failures, but require *ex vivo* culture and often self-renewing HSC divisions for genetic integration of the therapeutic gene.
 Totipotent stem cells with the ability to form all cell types are the result of fertilization. Pluripotent stem cells in the blastocyst stage embryo can grow into any of the approximately 200 cell types in the body. These can be isolated to generate embryonic stem cell lines (136) and have evoked much interest as they can potentially produce cells for therapeutic purposes. Due to their potency, differentiation needs to be tightly regulated to avoid uncontrolled growth that would otherwise result in teratomata (136). Multipotent stem cells, such as the HSCs, can only produce cells of a closely related family (e.g. blood cells). Their descendants, the multipotent progenitor cells, are further limited in their potential and produce only one or a few cell types. Multipotent stem cells are usually tissue-specific and have been described to varying degrees for the adult BM, nervous system, intestine, liver, pancreas, skeletal muscle, epidermis and the heart (for review see reference 137), but by far the most well characterized are the HSCs in the BM due to their accessibility and transplantability (138).

Regeneration through somatic plasticity is a common feature in invertebrates like amphibians, hydras and flatworms. In these, cells neighboring an injury de-differentiate into a pluripotent stem cell-like state, proliferate rapidly and differentiate again to regenerate the damaged or lost tissues, such as organs or limbs (139). Some species like planarians have instead clusters of pluripotent cells within their body, which migrate to the injured site. The mechanisms for how polarity, positional identity and the scale and proportion of the regenerating tissues are regulated are yet unresolved. Tissue regeneration is far more limited in most vertebrates, but for example the human liver retains some degree of regenerative ability throughout life.
TRANSDIFFERENTIATION

The hierarchical view of mammalian stem cell commitment, with irreversible loss of lineage potential during development, was recently challenged by several studies (for review see references 137, 140, 141). Multipotent tissue specific-stem cells were suggested to migrate to damaged organs to regenerate tissues by conversion of phenotypic as well as functional characteristics to cells in that particular organ. Such transdifferentiation into cell types normally not produced from a certain cell would depend on extrinsic regulation, from the new local environment, that direct differentiation in a fashion similar to regeneration in invertebrates (142, 143). At one point the whole concept of stem cells was questioned, as it was suggested that rather than referring to a distinct cellular entity, a stem cell most accurately should be referred to as a biological function, inducible in various cell types (141).

For many reasons, most studies investigated the transdifferentiation potential of HSCs. Typically, HSC- or BM-derived contribution to regeneration was evaluated in genetically conditioned or physically lesioned recipients. Cells from sex mismatched or transgenic (GFP or lacZ) donors were transplanted either directly into the tissues or via intra venous injection following lethal irradiation to reconstitute the whole hematopoietic system. In such experiments, BM cells were claimed to contribute to liver (144, 145), heart (146-148), skeletal muscle (149-151), central nervous system (152-154), pancreas (155, 156), epithelium (157) and vascular endothelium (158, 159), but most often at very low frequencies (typically <1% of all cells). Other studies also suggested a rare hematopoietic potential from non-hematopoietic donor cells (160, 161).

From a therapeutic perspective, the expectation was that any stem cell might turn into any tissue given the appropriate conditions. Such an optimistic idea drove clinical studies prematurely (162) before the mechanisms behind the reported functional improvements after transplantation of BM cells were understood (163-165). In addition such transplantation has potentially
unfavorable outcomes, as both heterotypic cell fusion (see next section) (166) and aberrant differentiation (167, 168) has been observed and might result in acute organ failures.

The initial hype of stem cell plasticity was dampened by several reviews and follow up studies in which higher criteria for evidence of transdifferentiation (137, 169) and lack of reproducibility (170-177) questioned the reliability of the earlier published studies (Figure 8). In some cases the primary reports were even shown to be incorrect with regard to their technical performance or the interpretation of data (170-173, 178-183) and yet other studies provided mechanisms, such as heterotypic cell fusion, as reliable explanations for the observed phenomena (173, 184-190). Given the rarity with which these events have been detected in vivo, very stringent criteria should be required for the demonstration of genuine transdifferentiation. Thus, the
reliability and robustness of the phenomena must be firmly established before
discarding basic concepts of developmental biology. Donor cell populations
should be prospectively isolated and transplanted without intervening culture
manipulations that may affect their gene expression profile and chromatin
configuration. Clonal analysis should be used to exclude the possibility of
bipotent differentiation due to heterogeneous donor cell populations.
Transplanted cells, with donor-specific markers, should give rise to robust and
sustained engraftment in the target tissues, and the frequency of conversion
should to be accurately quantified. Transition of one specific cell lineage into
another needs to be addressed by proper integration of the engrafting cells in the
target tissue and conversion to a tissue specific phenotype different from the
origin. To rule out artificial conversions of phenotype, the reprogramming of the
cell needs to be confirmed on gene expression level in silencing of gene
products characteristic of the original tissue and activation of genes specific to
the new cell fate. Functional effects of any conversions have to be established,
preferably on both cellular and whole organ level. Finally, following the recent
discovery of heterotypic cell fusions in experimental conditions, such
mechanism has to be ruled out as a possible explanation for the apparent
conversion.

Currently, according to these stringent criteria, no study has convincingly
documented a true transdifferentiation event (137) but despite this the debate
continues as to the plasticity of differentiation from multipotent stem cells (191-
196).

HETEROTYPIC CELL FUSION
Cell fusion is the process in which cells merge by joining their plasma
membranes forming a hybrid cell with two nuclei (197). Perhaps the best-
known hybrids are hybridomas, which are made by fusing myeloma cells with
lymphocytes to produce monoclonal antibodies. Cell fusion in live organisms is
a complex process, including cell recognition, adhesion and membrane merging and seems to have similar but unresolved mechanisms in disparate cell types (197, 198). Proteins or molecules involved in the merging of cell membranes are called fusogens. Given their widespread expression but the relative infrequency of fusion events in higher organisms, unidentified inhibitors might control the process or specific conditions such as cellular degeneration or regeneration might be needed (197, 198).

The importance of cell fusion during development and disease is emphasized by its involvement in a wide range of biological processes, including fertilization through fusion of the egg and sperm cells (199), the development of syncytium in muscle (200), bones (201) and placenta (202) as well as in the immune response (203) and during tumorigenesis (166).

Cells formed by heterotypic fusion of unrelated cell types (i.e. heterotypic cells) are called heterokaryons. Whereas syncytia arise spontaneously in normal tissues, heterokaryons have only been found during experimental conditions in vitro (187, 188) but recently also during tissue regeneration in vivo (184-186). The progeny of HSCs have been shown to fuse with a wide variety of target cells, including cardiomyocytes (173, 186), Purkinje neurons (186, 204, 205), hepatocytes (184-186) and muscle fibers (206, 207), resulting in a genetic and phenotypic conversion of the hematopoietic cell. Such fusion events can involve functional conversions on both the cellular and organ levels (145, 184, 185), but might not always result in changes of protein expression patterns (208). Whereas normal formation of hybrid cells usually results in divisionally inactive cells, the proliferative capacity of heterokaryons might be different (145, 209). Little is known about the mechanisms of heterotypic cell fusion, whether it is a regulated process or a random event. The fusogenic BM-derived cells has a HSCs origin (206, 207), but their identity as fusion partners are still largely unknown. However, cells of the myeloid lineage or even macrophages
have been involved in heterokaryon formation in both liver (190) and skeletal muscle (189, 210).
ASSAYS TO STUDY HEMATOPOIETIC STEM CELLS

When studying HSCs two main properties have to be evaluated, multipotent and self-renewing ability. The LT-HSC is the only hematopoietic cell that has both these properties, and therefore any cell population, or preferably cell clone, must provide long-term production of all mature blood cell types as well as new HSCs to be identified as a HSC.

PURIFICATION OF HEMATOPOIETIC STEM CELLS

The HSCs constitute a rare population of cells within the BM with an estimated frequency of less than one in 15,000 cells (44). To evaluate multiple properties of any BM cell population, the potential HSC needs to be enriched at least 1,000 fold and in some cases preferentially to a single cell (clonal) level. Unfortunately, the HSCs have no distinct size or molecular marker that can be used to purify them to this level (211, 212). With the development of monoclonal antibodies and fluorescent activated cell sorting (FACS) several markers have been established that can be used in combination to enrich for LT-HSCs within the hematopoietic organs (Figure 9). Mouse HSCs lack expression of cell membrane protein markers that are normally expressed by mature blood cells (e.g. B220, CD4, CD5, CD8, Mac-1, Gr-1 and Ter119). Among these lineage marker (lin) negative cells, a fraction of cells expressing both the receptor for stem cell factor (c-kit) and the stem cell antigen (Sca-1), contain all HSC activity in the BM (213). This population can be further subdivided into populations with distinct functional properties based on the expression of the adhesion molecule CD34 (46) and the Flt3 ligand C-fms like tyrosine kinase 3 (Flt3) receptor (43, 76, 77). The LT-HSCs express neither of these proteins (46, 77) but up-regulate expression of CD34 as they lose long-term self-renewing ability. The CD34 expressing cells (ST-HSC) have limited self-renewing ability
but are still multipotent (43) and produce LMPPs that start to express the Flt3 receptor (43, 76). The phenotypes of these cell populations change upon activation, during ontogeny and during aging (212, 214-216) and therefore purification based on functional properties or unique HSC markers would be favorable.

Staining of BM cells with the dyes **Hoechst 33342** and **Rhodamin 123** (217) results in heterogeneous labeling (43, 45) due to the differential exclusion of the dyes by multi-drug resistance membrane transporters which are highly active in HSCs (218). Despite a direct correlation between Hoechst 33342 efflux capacity and HSC identity in adults (219, 220) purification of BM cells based on the activity of these transporters can only enrich for HSCs and needs to be combined with phenotypic markers to reach high purities (43) or to compare HSCs identities based on developmental and activation status (220).

Recent findings suggests that **SLAM proteins** could be useful as markers to distinguish between HSCs, progenitor cells and mature cells, and that SLAM

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**Figure 9.** Strategy to purify hematopoietic stem and progenitor cells through FACS. Numbers indicate the frequency of each boxed population of total BM cells.
proteins expressed uniquely on these cell types can be used as single markers for purification (221). If these findings stand, they will revolutionize experimental HSC research in mice by allowing simple FACS identification and purification as well as detection of HSCs in situ within the BM.

EVALUATION OF HEMATOPOIETIC STEM CELLS

When evaluating HSCs, detection of their unique self-renewing and multipotent capacity is crucial. The later can be done in vitro by assaying differentiation capacity in various exogenous growth conditions such as media, serum and growth factors. Favorable are co-cultures with immortalized stromal cell lines that influence the stem cells by cell-to-cell contact and growth factor release (e.g. OP9/OP9Δ) (222). Since the readout of such cultures is the production of mature cells, they can at most give qualitative information of potential, as both stem and progenitor cells read out.

Since in vitro culture systems are limited to establishing lineage potential, in vivo models that also support and address self-renewal are favorable. BM populations with varying degrees of HSC enrichment can be evaluated by transplantation into myeloablated host animals by either intra venous or direct intra femoral injection. Qualitative and quantitative evaluation of engraftment from donor cells is determined by using congenic mouse strains (Figure 10). These differ only in the expression of the CD45.1 and CD45.2 isoforms (223, 224) and therefore allow transplantation without graft rejection but identification of donor and recipient cells. Qualitative engraftment can be evaluated by FACS analysis of antibody stained peripheral blood or BM cells to detect the number of donor cells (e.g. CD45.2+) and their contribution toward the different blood cell lineages (myeloid: Mac-1+, B cell: B220+, T cell: CD4+/CD8+) relative to the recipient cells (e.g. CD45.1+). Quantitative evaluation of the number of HSCs in cell populations can be assayed through co-transplantation with a reference population with a known number of HSCs that competes with the test
cells for host reconstitution. Such competitor cells can also provide a supportive function and short-term protection of the myeloablated host when test cells with low HSCs numbers are transplanted. The critical self-renewing capacity of donor cells can be assayed through serial transplantation of BM from primary recipients into secondary myeloablated recipients as HSCs in the test population need to undergo self-renewing cell divisions to reconstitute the BM of the secondary recipient.

With the discovery of the specific HSC-supporting BM microenvironment, the HSC niche, much interest has been focused on investigating the action of HSCs in situ. To date, however, no BM explant cultures have been established that allow evaluation of the interactions of HSCs with their microenvironment over time, and researchers are therefore limited to investigating histological

![Figure 10. In vivo functional evaluation of transplanted CD45.2 donor and CD45.1 competitor populations by FACS analysis of blood reconstitution. Lineage distribution of reconstituting donor cells is evaluated by staining for markers specific for myeloid (Mac-1), B (B220) and T (CD4/CD8) cells.](image)
specimens by immunohistochemistry. Such analysis is restricted by the limitation of phenotypical HSC markers and the low frequency of HSCs in the BM (211, 221). Models to evaluate HSCs in situ in living animals are developing, and will play an important role in investigating HSC migration and regulation (225-229).
FOCUS OF THE PRESENT STUDIES

The focus of this thesis is on two unique defining properties of HSCs, namely their multilineage potential and relative quiescence. Recent studies have suggested (96, 141) that HSCs, taken out of their quiescent state in the BM, may act differently and acquire new fates or lose properties that normally define them as stem cells.

First, early in vertebrate embryonic development partitioning into the three embryonic germ layers, ectoderm (skin and neural lineages), mesoderm (blood, bone, muscle, cartilage, and fat), and endoderm (respiratory and digestive tracts), takes place. This results in irreversible specification of all subsequently arising cells throughout adulthood. However, recent experiments have challenged this idea and suggest that stem cells under certain circumstances transdifferentiate into a much wider spectrum of progeny than previously anticipated. This theory of stem cell plasticity overturned our fundamental concepts of developmental biology and proposes that, given the right conditions, HSCs are able to respond to a variety of micro-environmental regenerative cues to become pluripotent.

Second, the quiescence of HSCs is one of their defining properties. It is thought to be important to avoid exhaustion of the stem cell compartment throughout life and to maintain genetic integrity over time. In several studies, the quiescent state of HSCs has been coupled with their stem cell function, suggesting that HSCs lose stem cell properties such as multilineage potential, self-renewing ability, and the ability to home to the BM stem cell niche as they enter the cell cycle.

The overall aim of this thesis has been to evaluate both stem cell plasticity (Paper I and II) and cell cycle related changes of HSC properties (Paper III) and establish both their significance and validity.
Transplantation of BM cells or enriched HSCs into adult recipients have been claimed to result in not only hematopoietic reconstitution but also the regeneration of a variety of non-hematopoietic lineages in multiple organs through transdifferentiation (for review see reference 137). The recently reported ability of BM cells to regenerate cardiomyocytes upon transplantation into infarcted myocardium were unprecedented, with regard to both the levels of potential transdifferentiation observed (147) and improved cardiac function (148). Based on these studies, but without further evaluation of the function or duration of potentially BM-derived cardiomyocytes, a number of clinical trials have been initiated world-wide, in which patients that suffered from acute myocardial infarction have been transplanted with autologous BM cells (163-165).

Follow up studies of BM derived transdifferentiation have questioned its significance and even existence, and have provided alternative mechanisms for the reported findings. It has been shown that BM-derived contribution to non-hematopoietic cell lineages do occur but through cell fusion rather than transdifferentiation, and moreover that such heterotypic fusion might occur spontaneously (186, 190, 205-207). However, as none of these findings were from steady-state physiological conditions, it remained to be established whether heterotypic fusion is a normal blood cell property or only a result of organ failure or tissue injury. Considering the low frequency of these events (<1%) it is unlikely that heterotypic fusion can significantly improve organ function unless the resulting heterokaryon have a selective advantage. The functional consequences of heterotypic fusion need to be investigated in more detail, particularly in light of the known association of cell fusion in transformation and metastasis in cancer (166).
AIMS
We aimed to evaluate the ability of BM cells to regenerate the infarcted myocardium through transdifferentiation. Specifically, we applied different murine acute cardiac injury and BM transplantation models to establish in detail the extent and durability of any BM-derived engraftment, the identity of engrafting donor cells, and the cellular mechanisms responsible for the reported developmental plasticity. Furthermore, we wanted to establish if heterotypic fusion of BM cells and cardiomyocytes, Purkinje neurons, hepatocytes or skeletal muscle fibers occurred spontaneously during development or was a result of experimental conditions. We also sought to identify the origin of the BM-derived fusion partners and the mechanisms behind this phenomenon.

SUMMARY
Our study confirmed that BM-derived hematopoietic cells engraft following direct transplantation into the myocardial infarcted heart (Paper I). Such engraftment was highly selective for the damaged myocardium but transient and virtually lost four weeks after transplantation. Engrafting donor cells had phenotypical and morphological characteristics of myeloid cells, demonstrating that hematopoietic cells rather than cardiomyocytes accounted for the transient engraftment previously reported (147).

We also investigated the cardiomyocyte potential of BM cells following cytokine-induced mobilization of BM cells to the blood and infarcted myocardium (Paper I). This strategy resulted in higher and more sustained levels of BM-derived engraftment in the infarcted myocardium compared to direct injection. Engrafting cells had the same phenotypical and morphological characteristics of myeloid cells consistent with earlier findings of invasion by inflammatory cells during scar formation in mice (230). However, the involvement of HSCs in the normal repair process is brought into question, as
we did not observe any hematopoietic stem or progenitor cell mobilization to the blood in response to acute myocardial infarction.

In contrast to the infarcted myocardium, we consistently observed a very low number of BM-derived cells in the viable myocardium with a phenotype and morphology supporting a cardiomyocyte identity. By using different donor (GFP) and recipient (lacZ) transgenic marker genes (231, 232) we conclusively demonstrated that these rare cells were derived through heterotypic fusion with host cardiomyocytes and donor hematopoietic cells (Paper I). Such events were never observed in mice in which BM cells were transplanted directly into the infarcted myocardium. The requirement for non-direct BM transplantation in the fusion process might be explained by a higher and sustained delivery of hematopoietic cells to the infarcted heart from reconstituted BM, or by the lethal irradiation conditioning prior to transplantation.

Taken together, these studies suggested that BM derived cells contribute to non-hematopoietic cell lineages through heterotypic cell fusion rather than transdifferentiation. Others have suggested that such fusion might occur continuously in steady state adult tissues with various cell types (186, 190, 205-207). However, as none of the models previously used have reflected steady state physiological conditions, it remained to be established if heterotypic fusion is a normal blood cell property. We addressed this by transplanting BM cells into adult c-kit receptor deficient (c-kit<sup>W41/W41</sup>) mice which, due to an intrinsic HSC deficiency, allow significant lympho-myeloid reconstitution (233) from wild type HSCs in the absence of myeloablation (Paper II). Despite stable multilineage hematopoietic reconstitution from donor derived cells, these failed to provide any contribution toward cardiomyocytes, Purkinje neurons, hepatocytes or skeletal muscle fibers. In contrast, when tissue injury (myocardial infarction, striatal toxin treatment, skeletal muscle toxin- or cryo-induced damage or whole body lethal irradiation) was inflicted, BM-derived heterotypic fusion with these cell types was readily detected in all mice.
The BM-derived fusion partners have been reported to have a HSC origin (173, 190, 206, 207) and are at least in some cases of the myelomonocytic lineage (189, 190, 206, 210), but the exact identity of the hematopoietic cells with fusogenic abilities has not been established. We evaluated myeloablated mice with myeloid-restricted reconstitution and detected donor contribution to cardiomyocytes, hepatocytes and skeletal muscle fibers. The lack of contribution to Purkinje neurons suggested that BM-derived fusion partners to Purkinje neurons might have a non-myeloid origin. To investigate this, myeloablated mice with lymphoid-restricted reconstitution were evaluated and in these, donor contribution to all four cell types (including Purkinje neurons) was observed (Paper II).

Although our studies in adult mice supported the fusion of lymphocytes with non-hematopoietic cells upon insult, they do not prove a role for heterotypic cell fusion during steady-state conditions. As homotypic cell fusion plays a role in normal organ development, we investigated whether heterotypic fusion with lymphocytes might contribute toward development of non-hematopoietic lineages during embryonic development (Paper II). In utero transplantation of severe combined immuno-deficiency (SCID) recipients (234) allow lymphoid reconstitution without myeloablation. In these, both donor-derived hepatocytes and Purkinje neurons, with characteristic morphology and lineage marker expression, but no donor-derived cardiomyocytes or skeletal muscle fibers were detected. These lymphocyte-derived hepatocytes and Purkinje neurons were not derived from transiently circulating infused donor BM cells (including myeloid cells), but rather the lymphoid progeny of reconstituting HSCs. Lower or undetectable levels of lymphocyte-derived hepatocytes and Purkinje neurons in transplanted steady state neonatal and adult SCID recipients demonstrated that this unique ability of lymphocytes is restricted to a narrow window during fetal development.
CONCLUSIONS

Our studies were in agreement with two other reports, published at the same time, of the inability of transplanted hematopoietic cells to transdifferentiate into cardiomyocytes following myocardial infarction (171, 172) and established that BM donor cell engraftment was entirely of hematopoietic and transitory nature, and that no BM-derived cardiomyocytes could be observed in the damaged myocardium (Paper I). This suggests that the observed enhanced survival and improved cardiac function following BM mobilization of infarcted mice (148) is likely to be mediated through a mechanism distinct from transdifferentiation of BM cells to cardiomyocytes. In contrast, cardiomyocytes fused with BM-derived cells could be observed outside of the infarction area, but were too rare to be of any functional relevance. Our findings challenge the experimental basis for the ongoing extensive clinical BM transplantation trials of myocardial infarction patients (163-165), as these trials were largely initiated on the assumption that BM-derived cells could generate high levels of cardiomyocytes through transdifferentiation (146-148). In addition, the identity and fate of transplanted BM cells has to be established as we and others have found adverse outcomes (167, 168) as a consequence of engrafting donor cells. Finally, if BM cells can contribute to any indirect functional improvement, such as promoting angiogenesis or preventing cardiac remodeling following myocardial infarction (235-237), the mechanisms for such effects have to be identified before translating into treatments of patients.

By studies of uninjured mice, we established that detectable in vivo heterotypic fusion of BM-derived cells with cardiomyocytes, Purkinje neurons, hepatocytes and skeletal muscle fibers does not occur in steady state adult tissues, but is stimulated by lethal irradiation or organ specific injuries (Paper II). These findings question previous studies implicating that BM-derived fusion might occur normally in healthy individuals (186, 190, 206, 207). Since replacement of the blood system of recipient mice in these studies was
invariably achieved following lethal whole body irradiation (173, 184-186, 189, 190, 205-207, 210, 238), we argue that the observed cell fusion events might have been a consequence of irradiation-induced effects. We also demonstrate a previously unrecognized role of lymphocytes as partners for heterotypic fusion and provide compelling evidence for lymphocyte contribution to hepatocytes and Purkinje neurons during embryonic development. These data demonstrate for the first time that the hematopoietic system might contribute toward non-hematopoietic cell lineages during normal organogenesis. Using advanced cre-lox lineage-tracing experiments we are currently further characterizing such spontaneous heterotypic fusion during normal development. Our preliminary data support both that such processes occur at a significant level and are truly a result of heterotypic cell fusion. However, the efficiency by which blood derived cell fusion occur in our and other studies is very low, and a therapeutic exploration of this phenomena will require insight into mechanisms that can enhance the fusion efficiency, or that fusion occurs with a target cell with considerable proliferative potential and a competitive advantage over other cells of that lineage (145, 184, 185).

HEMATOPOIETIC STEM CELL CYCLE (PAPER III)

HSCs in steady state BM cycle rarely and are mainly in the G0 phase of the cell cycle (47, 48). It was previously believed that only a rare number of mouse LT-HSCs contribute actively to hematopoiesis in steady state adult BM (239), but more recent studies have showed that virtually all adult LT-HSCs cycle within a period of 4-8 weeks (47, 48). Mechanisms must be in place to dramatically enhance the speed of proliferation of LT-HSCs on demand, such as following infections, hemorrhage, BM transplantation or during fetal development when HSCs expand extensively (61, 240). Studies of highly purified BM HSCs in
vitro have clearly demonstrated that LT-HSCs need considerably longer time to complete their first cell division compared to more committed progenitors. This might be explained by their unresponsive G₀ quiescent state that requires entry into G₁ and longer time for preparation of the cell cycle machinery. Subsequent divisions of HSCs are much shorter, as they no longer need to first prepare for G₁ entry, and have been assumed to have a transit time comparable to progenitors (54, 63, 65, 241). However, previous in vitro studies have been complicated by the fact that while current ex vivo expansion conditions dramatically expand cell numbers, they only maintain or at best slightly expand LT-HSCs over time (98, 99, 101, 102, 242-245), resulting in cultures wherein the vast majority of cells are committed progenitors rather than LT-HSCs.

The current view that HSCs in S/G₂/M phases of the cell cycle are compromised in their ability to short- and long-term reconstitute recipient mice upon transplantation (63-66, 96) has partially been based on the assumption that actively proliferating LT-HSCs have a cell cycle transit time identical to that of committed progeny and that HSCs are identifiable by their phenotype alone. Therefore, the inability to uniquely identify LT-HSCs prospectively (211) complicates and limits the interpretations of these studies.

AIMS
To directly establish the cell cycle kinetics of actively proliferating LT-HSCs by using ex vivo expansion (99) and the extensive expansion of HSCs during normal fetal development (240) as model systems.

SUMMARY
Our studies of adult HSCs, fractionated into non-dividing (G₁) and dividing (S/G₂/M) cells and expanded ex vivo, suggested that HSCs in the S/G₂/M phases do not gain repopulating activity as they re-enter G₁ in culture (Paper III). Likewise, HSCs in G₁ do not lose significant HSC potential when they divide in...
cultures. These data are most compatible with the original S/G2/M cells being mainly composed of actively dividing short-term stem or progenitor cells whereas LT-HSCs, due to a prolonged G1 cell cycle transit, are mainly present in the G1 fraction. To obtain further support for this we used methods for viable cell division tracking and obtained direct evidence that the cell cycle transit time of ex vivo self-renewing LT-HSCs is prolonged compared to their committed progeny, during not only the first but also subsequent cell divisions. In addition, also ST-HSCs have a prolonged cell cycle transit relative to that of committed progenitors, although shorter than that of LT-HSCs.

We also investigated the cell cycle kinetics of HSCs under physiological conditions in vivo by studying the fetal liver at a stage of development when HSCs expand extensively. These studies confirmed and extended the conclusions reached through the ex vivo expansion models. Although all hematopoietic progenitor cells and HSCs in 14.5 dpc liver had proliferated within 48 hours, they displayed dramatically different cell cycle profiles and kinetics of BrdU incorporation. Actively proliferating fetal HSCs passed through a stage of relative quiescence and prolonged G1, resulting in a doubling of the cell cycle transit time when compared to hematopoietic progenitor cells.

CONCLUSIONS
We have demonstrated that the enrichment of LT-HSC activity in the G0/G1 phase of the cell cycle, even when actively proliferating in vitro and during fetal development, does not necessarily reflect cell cycle specific effects on HSC function, but are at least in part rather an effect of a protracted G0/G1 transit (Paper III). We propose that this is a unique and defining property of HSCs, regardless of the stage of development and pathways promoting HSC expansion. The conclusions from our studies could only be reached by identifying and evaluating LT-HSCs through their function, as there is no reliable phenotype of LT-HSCs following in vitro and in vivo expansion (212, 214). Thus, previous
studies implicating cell cycle specific effects on LT-HSC engraftment (63-66) as well as gene expression (246), should be re-evaluated in light of the fact that functionally defined LT-HSCs, due to a prolonged cell cycle transit, always are enriched in G1 relative to S/G2/M. Although our studies do not exclude cell cycle specific effects on stem cell function, they do establish that the enrichment of proliferating HSCs in G1, at least in part, is due to their extensively protracted transit through this phase of the cell cycle.

We propose that this distinct regulation of the cell cycle in HSCs could be a requirement for their self-renewal (57) and limit their proliferative capacity to avoid exhaustion of the LT-HSC compartment (74, 75). Thus, it will be of considerable interest and importance to identify the unique regulatory mechanisms of HSC cell cycle transit during development as well as in steady state adult hematopoiesis.
Konstant produktion av blodceller i benmärgen krävs för att bibehålla normal syretransport i blodet, sårläkning och ett effektivt immunförsvar. Bakom denna produktion ligger hematopoetiska blodstamceller som regleras av en komplex interaktion mellan dem och deras omgivning. Varje blodstamcell kan med självdöende celldivisioner, där minst en daughtercell bibehåller en stamcellidentitet, producera alla typer av blodceller och bidra till blodbildning genom hela livet. De är företrädesvis lågaktiva i benmärgen, men har i flera avseenden tillskrivits unika egenskaper i situationer då de sätts ur sitt normala tillstånd. Det är några av dessa situationer denna avhandling berör.

De senaste åren har blodstamceller i flera studier ansetts bidra till andra celltyper än blodceller. Benmärgsceller, anrikade för blodstamceller, har planterats till olika modeller av organskada i möss med förhoppning om att regenerativ miljö i skadad vävnad ska stimulera dem att bidra till bildning av andra celler än blodceller. De initiala fynden från sådana experiment har skapat stora förväntningar på användning av blodstamceller för cellterapi på patienter med olika degenerativa sjukdomstillstånd. Från våra studier av möss med experimentellt inducerad hjärtinfarkt kan vi konstatera att blodstamceller saknar potential att bilda hjärtmuskelceller och att den enda mekanism med vilken blodceller bidrar till hjärtmuskel är genom fusion med överlevande hjärtmuskelceller. Dessa fynd kullkastar underlaget för pågående kliniska studier samt storskalig etablering av benmärgsinjektion i hjärtat som behandlingsmetod för patienter med hjärtinfarkt. Hur hybrider av två olika ursprungscarceller bildas eller vilken biologisk relevans de har återstår att visa. Eftersom bildning av dessa sker med låg frekvens och endast efter kraftig skada i den studerade vävnaden, talar mycket för att fusion mellan blodceller och andra celler inte är ett normalt fenomen med biologisk betydelse i vuxna
individer. Å andra sidan visar vi att blodstamceller transplanterade till möss tidigt under fosterutvecklingen kan bidra till bildning av hepatocyter i levern och Purkinjeceller i lillhjärnan. Uppenbarligen kan blodstamceller under en kort period av fosterutvecklingen inverka på normal utveckling av dessa organ. Dessa primära fynd måste noggrant reproduceras och karaktäriseras och mekanismerna bakom cellfusion måste identifieras innan vi vet om de är ett resultat av en naturlig biologisk process som går att manipulera och utnyttja för terapeutiska syften.

Vanligtvis är stamceller inaktiva, vilket innebär att de är mindre mottagliga för stimulans av tillväxtfaktorer och inte delar sig för att bilda nya stamceller eller blodceller. Under vissa betingelser, som vid infektioner eller blödningar då kraftig ökning av antalet blodceller krävs, kan de dock exponentiellt öka bildningen av blodceller. Att bibehålla en inaktiv status är förmodligen en nödvändig egenskap för att bibehålla livslång blodcellsproduktion och det har därför antagits att aktiv celldelning av blodstamceller leder till reducerad stamcellsförmåga. Detta antagande baseras på experimentella observationer som antyder att stamceller som delar sig har sämre stamcellsegenskaper än de som är inaktiva. Vi har studerat detta och kan visa att celldelning inte leder till förlust av stamcellsförmåga samt att den observerade skillnaden mellan anrikade blodstamceller i inaktivt och aktivt tillstånd beror på en för dem unik reglering av celldelningscykeln. Detta resulterar i en långsammare delningscykel och därmed en huvudsaklig distribution av blodstamceller bland icke delande celler. Dessa fynd är av stor vikt för att förstå och vidare undersöka några av de definierande egenskaperna hos blodstamceller, nämligen att självförnya sig och bibehålla blodbildning genom livet.


Critical role of the cytokine tyrosine kinase receptor Flt3 in fetal and adult T lymphopoiesis. Sitnicka E, Buza-Vidas N, Ahlenius H, Cilio CM, Gekas C, Nygren JM, Svensson M, Agace WW and Jacobsen SEW. Submitted

Failure of transplanted bone marrow cells to adapt a pancreatic β-cell fate. Taneera J, Rosengren A, Renstrom E, Nygren JM, Serup P, Rorsman P and Jacobsen SEW. Submitted

Lack of functional improvement following transplantation of bone marrow cells to the infarcted myocardium. Roell W, Breitbach M, Nygren JM, Jacobsen SEW, Fleischmann BK. Manuscript in preparation

The cytokine signaling inhibitor Lnk is a potent physiological negative regulator of hematopoietic stem cell self-renewal. Buza-Vidas N, Nygren JM, Jensen CT, Qian H, and Jacobsen SEW. Manuscript in preparation

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REFERENCES


