Developmental Origins of the Murine Hematopoietic System

De oorsprong van het hematopoietische systeem
tijdens de ontwikkeling van de muis

Proefschrift

ter verkrijging van de graad van doctor
aan de
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To my parents and Onno.
Dla moich rodziców i Onno.

Contents

LIST OF ABBREVIATIONS

AGM	**A**orta-**G**onad-**M**esonephros
AML	**A**cute **M**yeloid **L**eukemia
BM	**B**one **M**arrow
CFU-C	**C**olony **F**orming **U**nit-**C**ulture
CFU-S	**C**olony **F**orming **U**nit-**S**pleen
DNA	**D**eoxyribo**N**ucleic **A**cid
DPC	**D**ay of **P**ost **C**oitum
E	**E**mbryonic day
EGFP	**E**nhanced **G**reen **F**luorescent **P**rotein
EYFP	**E**nhanced **Y**ellow **F**luorescent **P**rotein
FACS	**F**luorescence **A**ctivated **C**ell **S**orting
FCS	**F**etal **C**alf **S**erum
FL	**F**etal **L**iver
GFP	**G**reen **F**luorescent **P**rotein
HIAC	**H**ematopoietic **I**ntra**A**ortic **C**luster
HSC	**H**ematopoietic **S**tem **C**ell
IL	**I**nter**L**eukin
kB	**k**ilo **B**ase
kD	**k**ilo **D**alton
LN	**L**ymph **N**ode
LTR	**L**ong – **T**erm **R**epopulating
mRNA	**m**essenger **R**ibo**N**ucleic **A**cid
PBS	**P**hosphate **B**uffered **S**aline
PCR	**P**olymerase **C**hain **R**eaction
P-Sp	**P**ara-aortic **Sp**lanchnopleura
SAP	**S**ub**A**ortic **P**atch
SCL	**S**tem **C**ell **L**eukemia
SP	**S**pleen
YS	**Y**olk **S**ac

CHAPTER 1

General Introduction

Chapter 1 – General Introduction

1. Development of the hematopoietic system in the mouse

1.1. Adult hematopoietic system

The adult hematopoietic system is a complex cellular hierarchy that generates a diverse range of blood cells serving many different functions during the entire lifespan of an organism. These hematopoietic cells include erythrocytes – transporting oxygen and carbon dioxide, lymphocytes (B- and T) essential for the immune response and granulocytes (basophiles, eosinophils and neutrophils), which are able to phagocytose dead cells or foreign invading microorganisms/foreign bodies and taking part in inflammatory responses. Other types of hematopoietic cells found in the adult are monocytes, which can mature in macrophages, and platelets – cell fragments derived from megakaryocytes in the bone marrow, which are involved in blood clotting (Wintrobe's Clinical Hematology, 1998) (**Fig.1**). Interestingly, it was estimated that during the entire mouse lifespan (typically 2 years) a tremendous number of hematopoietic cells is generated, comparable to 60% of the mouse body weight and an amplification (between HSC and circulating blood cells) between 70 000 up to 720 000 (Mackey et al., 2001). The major lineages of hematopoietic cells represent according to Nečas et al. (1998) erythrocytes and granulocytes.

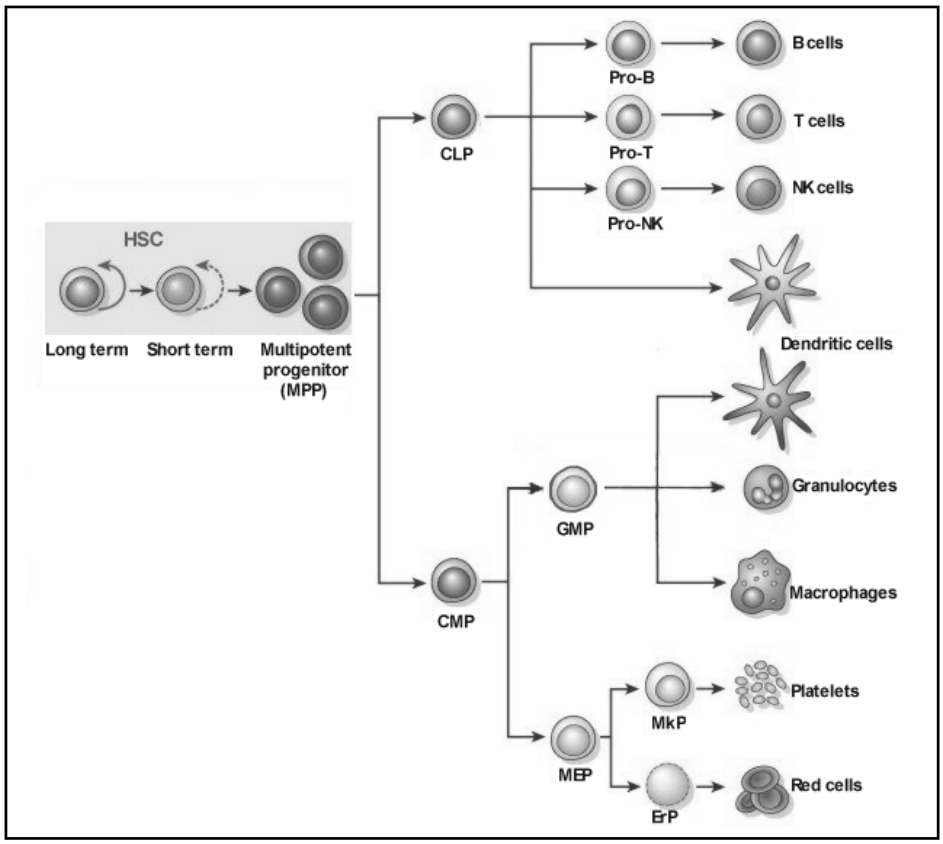

Figure 1. Hematopoietic hierarchy in adult. In the present model of adult hematopoiesis the hematopoietic stem cell (HSC) with long-term potential gives rise to cells with short-term potential. Both are able to self-renew (as indicated by curved arrows). In turn, HSCs differentiate into multipotent progenitors, which give rise to CLP (common lymphoid progenitor) and CMP (common myeloid progenitor); CLP is a committed progenitor for all lymphoid cells (however CLP existence and/or its nature are still debatable-Katsura et al., 2002; Kondo et al., 1997), and CMP is a progenitor for all myeloid cells. Both can produce dendritic cells (adapted from Reya et al., 2001).

In the adult mouse, the predominant site of hematopoiesis is the bone marrow (BM). However, other organs such as spleen, thymus or lymph nodes also harbor hematopoietic activities. In addition, a variety of hematopoietic cells are also found in the circulation, although some cells such as macrophages and mast cells are strictly restricted to certain tissues. While in the BM a hierarchy of different types of hematopoietic cells can be found, starting from HSCs, multipotent and more committed progenitors up to mature cells, other hematopoietic organs are more specified with regard to the hematopoietic cell type. In the thymus, for example, T-cell precursors undergo maturation and selection into functional T-lymphocytes (Fundamental Immunology, Paul, 2003). Formed during the late fetal stage, the spleen becomes a reservoir for B- and T-lymphocytes and a place where blood destruction occurs. In addition, the spleen together with the lymph nodes and gut-associated lymphoid tissues belongs to peripheral lymphoid sites, where entering lymphocytes can be activated by specific antigens and induce an adaptive immune response (Immunobiology, Janeway et al., 2001).

The most informative description of the diverse array of hematopoietic cell types is found in a schematic picture of the hematopoietic lineage differentiation hierarchy. This hierarchy begins at the most important founder cell of the adult blood system, the hematopoietic stem cell (HSC). Hematopoietic stem cells primarily reside in the bone marrow (BM). Their hallmarks are not only their self-renewal ability, and their capacity for multilineage hematopoietic cell production sustained for the entire lifespan of the animal, but also their ability to reconstitute the blood system of adult irradiated recipients (Abramson et al., 1977; Uchida and Weissman, 1992). They represent a very rare (1-10 in 100 000 of murine BM cells), but also very heterogeneous population of cells, since no exclusive marker for the hematopoietic stem cell has so far been identified (Boggs et al., 1982; Abkowitz et al., 2000; Morrison and Weismann, 1994). From clonal genetic marking *in vivo* experiments it has been demonstrated that a single hematopoietic stem cell is able to give rise through gradual differentiation from multipotent hematopoietic progenitors to more committed progenitors to finally all terminally differentiated blood cells (Jordan and Lemischka, 1990; Smith et al., 1991; Cao et al., 2004).

According to the steady-state model of the adult hematopoietic system the number of HSCs remains constant under normal physiological conditions. It has been

shown that adult HSCs are largely quiescent with most of them in the G_0 / G_1 phase of the cell cycle (Lemischka et al., 1986). However, in situations of stress or damage, the mobilization of HSCs occurs, followed by an increase in the absolute number of HSCs in the blood circulation (Iscove and Nawa, 1997; Lapidot et al., 2003; Dzierzak, 2002). The purpose of the quiescent state of HSCs is thought to prevent exhaustion of the HSC pool, although some reports showed that the majority or even all long-term repopulating HSCs could actively (on average once every 2 months) proliferate (Fleming et al., 1993; Cheshier et al., 1999).

An interesting observation in the steady-state model is that, within the murine adult HSC population, HSC subsets with lineage-biased differentiation potential can be found. This class of stem cells, which is distinct from more primitive adult HSCs, is still able to give rise to all blood lineages, but with the skewed ratio of myeloid to lymphoid cells, which is maintained even through long-term, serial transplantations (Muller-Sieburg et al., 2004).

In addition, serial transplantation experiments showed that the composition of the adult BM HSC population is changing over the mouse lifespan with regard to different lineage-biased HSCs. Similar to human array of adult HSCs, the murine lymphoid-biased HSC, myeloid-biased HSC and the most primitive HSC subsets contribute altogether to adult hematopoiesis. As proposed by Muller-Sieburg et al. (2004), the lymphoid-biased HSCs contribute to hematopoiesis quite rapidly in contrast to the slow, but long-term contribution from myeloid-biased HSCs, which are predominant in aged mice and humans (starting from 21 years old) (Sudo et al., 2000; Muller-Sieburg et al., 2004). Moreover, in transplantation studies, where retrovirally marked HSCs were injected into adult irradiated recipients, the long-term analysis confirmed that the hematopoietic system of the recipient could be repopulated by a few HSC clones with variable lifespans. These data support the clonal succession model of hematopoiesis, where hematopoiesis is driven by a subset of active HSC clones and is replenished by new ones over time (Kay, 1965; Jordan and Lemischka, 1990; Keller and Snodgrass, 1990). The variable lifespans of HSC clones could possibly be explained by limited replicative potential and shortening of telomeres, which was observed in HSCs during serial transplantations in mice (Allsop et al., 2001; Allsop et al., 2003).

In the first step of differentiation HSCs give rise to MPPs (multipotent progenitors), which in turn gives rise to two main hematopoietic lineage progenitors: lymphoid (CLP) and myeloid (CMP) (Kondo et al., 1997; Akashi et al., 2000). CMPs further differentiate into more committed progenitors: megakaryocytic/erythroid (MEP) and myelomonocytic (GMP) (Akashi et al., 2000). Interestingly, another early multipotent progenitor, the lymphoid-primed multipotent progenitor (LMPP) has been described. It differs from HSCs by expression of Flt3, a cytokine tyrosine kinase receptor crucial for lymphoid commitment (Adolfsson et al., 2005; Sitnicka et al., 2002). This progenitor lacks megakaryocyte/erythroid potential. However, as has been shown for the CLP, which gives rise usually to lymphocytes - T, B and natural killer

cells (Kondo et al., 1997), it can be redirected to myeloid differentiation as demonstrated *in vitro* culture (Kondo et al., 2000). This redirection of CLP commitment may be related to proposed lineage-priming hypothesis, which is based on the observation that genes for differentiation into multiple lineages are simultaneously expressed in hematopoietic progenitors (Hu et al., 1997; Ye et al., 2003). A further explanation may be the instability of hematopoietic lineages where changes in a single regulatory lineage factor can influence the fate of progenitor commitment (Heavey et al., 2003; Enver et al., 1998).

Thus, the adult hematopoietic hierarchy functions as a continuum of expanding and differentiating stem cells committed to the production of the highly varied and functionally different progenitors and mature cells required for the viability and protection of the individual.

1.2. Hematopoietic hierarchy in adult versus embryo

Within the mouse conceptus, hematopoietic cells are *de novo* generated in highly vascularized regions. The hematopoietic system at these early stages of development is very dynamic with no recognizable steady state. Moreover it undergoes an intensive remodelling within different hematopoietic tissues as they are generated.

In contrast to the adult hematopoietic system, where all progenitors and mature blood cells arise from hematopoietic stem cells and can be categorized in the classical hierarchical scheme, in the mouse embryo the blood cells defy classification into such a differentiation hierarchy. This has also been found to be the case for the human embryo (Tavian et al., 2001; Dzierzak, 2002 review).

The evidence for hematopoietic cell lineage relationships in the embryo, starting with a hematopoietic stem cell to terminally differentiated hematopoietic cells, is still lacking. Moreover, it appears to be that hematopoietic hierarchy of embryonic blood system differs from the hierarchy found in the adult hematopoietic system. Indeed, the first hematopoietic cells, differentiated primitive erythrocytes, appear in the yolk sac blood islands long before the appearance of the first HSCs. The rapid production of primitive erythrocytes is required to support the dynamic development of the growing embryo by transporting oxygen and carbon dioxide more efficiently that by direct oxygen diffusion (Cumano and Godin, 2001). These large nucleated erythroid cells appear around E7-7.5, while the first adult repopulating HSCs arise later at E10.5 in the AGM region (Palis and Yoder, 2001; Müller et al., 1994; Medvisky and Dzierzak, 1996). Interestingly, many other hematopoietic progenitors and mature cells arise soon after the primitive erythrocytes arise in the yolk sac blood island. These include hematopoietic progenitors with erythroid/myeloid/lymphoid activities that appear at E8-8.5 (Palis et al., 2001; Palis and Yoder, 2001; Cumano et al., 1996).

In summary, one of the main differences between the embryonic and adult hematopoietic hierarchy is that during the embryonic stage hematopoietic progenitors and mature cells are present before HSCs. The relationship between the earlier-detected

progenitors and differentiated cells and the later-found HSCs still remains unclear. Hence, the hematopoietic system must be generated from cells that are not yet committed to the hematopoietic system several times during mouse development. Thus, the lineage relationships between these unknown cells and the resulting hematopoietic cells in the embryo must be elucidated.

1.3. Hematopoiesis in mouse conceptus

The hematopoietic system originates from mesoderm, one of the 3 germ layers produced in the gastrulation process. Gastrulation begins at E6/6.5 in the primitive streak (PS)-stage and is followed by the formation of the extraembryonic tissues and subsequently the tissues of the embryo proper (intraembryonic) (Dzierzak and Medvinsky, 1995 review).

As determined by lineage tracing studies, at early stages in the development of hematopoietic tissues, mesoderm spreads first laterally and then leaves the PS from the posterior side to contribute to the yolk sac (Lawson et al., 1991). Fate mapping data obtained by Kinder et al. (1999), also showed the PS-derived cells give a major contribution to the extraembryonic mesoderm and that the different temporal and spatial distributions of the mesodermal cells during gastrulation dictate the anteroposterior patterning of mesodermally-derived tissues, including hematopoietic. Moreover, the earlier appearance of erythroid precursors extending from the YS to the bulk of the vitelline endothelium may indicate an independent generation of hematopoietic and endothelial lineage (Kinder et al., 1999). However, it has been shown recently that a common precursor for both these lineages, the hemangioblast exists in the mouse embryo starting from mid-streak of gastrulation (Huber et al., 2004; Jaffredo et al., 2005).

During development of the mouse hematopoietic system the localization of the hematopoietic sites, where hematopoietic cells are generated or found, is changing as a function of developmental time (**Fig.2**). In addition, during mouse ontogeny there occur two different types of hematopoiesis: primitive and definitive. Primitive hematopoiesis generally takes place in the yolk sac, is transient (occurring only during these early developmental stages) and includes production of nucleated erythrocytes and macrophages (Barker, 1968). In contrast to primitive hematopoiesis, definitive hematopoiesis accounts for multilineage hematopoietic cell production that is self-renewable (Lee et al., 1991) and generally occurring intraembryonically. The yolk sac and placenta are the extraembryonic hematopoietic regions, where primitive and definitive hematopoiesis occurs respectively (Palis and Yoder, 2001; Dzierzak et al., 1998; Gekas et al., 2005; Ottersbach and Dzierzak, 2005). Intraembryonic hematopoietic sites such as the AGM and fetal liver, although initially harboring primitive hematopoietic cells (present only after the circulation is established) are the main reservoirs for the production and maintenance of definitive hematopoietic cells.

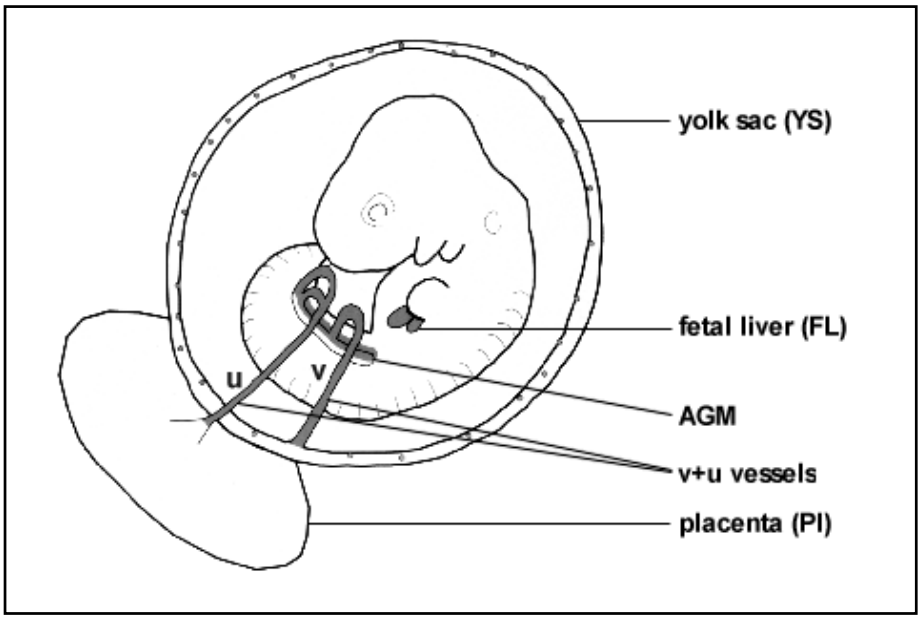

Figure 2. Schematic representation of hematopoietic sites of mouse conceptus at midgestation.
During mouse ontogeny the first site of blood production is the yolk sac, where primitive erythrocytes are generated in the blood islands starting from E7.5. In the intraembryonic part of the embryo, i.e. AGM and FL, definitive adult repopulating HSC are found at E10.5 and E11.5 respectively. Extraembryonic placenta and umbilical and vitelline vessels (v+u) also harbor definitive hematopoietic activity, which can be detected already at E10.5 (adapted from Dzierzak et al., 1998).

1.3.1. Extraembryonic hematopoietic sites

The developing mouse hematopoietic system is comprised of two hematopoietically active sites localized outside the developing embryo: the yolk sac and placenta. These tissues are connected with the embryo through the vitelline and umbilical vessels respectively. The first visible hematopoietic events during mouse ontogeny occur in the extraembryonic part of the conceptus. In the yolk sac between E7 and E7.5 aggregates of cells form blood islands, which give rise to the first primitive hematopoietic cells. These blood islands are multifocal with a punctate distribution throughout the yolk sac. Primitive erythroid cells are produced rapidly and in direct contact with surrounding differentiated endothelium (Maximow, 1909; Russel and Bernstein, 1966; Palis et al., 1999; Palis and Yoder, 2001; Ghatpande et al., 2002). The endothelium is shortly thereafter remodeled to form the vasculature of the yolk sac.

Formation of the yolk sac may differ between different species, particularly human and mouse. Indeed, turning movements during early mouse ontogeny result in the embryo body being completely enveloped by the yolk sac. In contrast the yolk sac of the human conceptus is maintained only as an extension and does not surround the human embryo body. In addition, in the mouse mesodermal cells originate from primitive ectoderm during gastrulation, while in the human, yolk sac mesoderm is thought to come from primitive endoderm (Enders and King, 1993). The significance of these differences is unknown.

The mouse yolk sac consists thus of mesoderm and visceral endoderm, which are very important for proper development of the embryo. For example, the visceral endoderm is involved in the transport and metabolism of maternal molecules, synthesis of serum proteins and plays important signaling role in formation of the blood island. The mesoderm, as already stated, takes part in the production of the first hematopoietic cells in the blood islands (Palis and Yoder, 2001).

In nonmammalian species, yolk sac analogs are found. In the amphibian species equivalent of the yolk sac is called the ventral blood island region (VBI), which is derived from ventral mesoderm (Ciau-Uitz et al., 2000). In vertebrate species such as chick, the blastodisc functions as a presumptive yolk sac analog. In the zebrafish, a frequently used developmental animal model system, the yolk sac or yolk sac analog is absent (Detrich et al., 1995; Bahary and Zon, 1998). However, apart from described differences between species, primitive hematopoietic development in general appears to be highly conserved. Recently, the murine placenta was also discovered as hematopoietically active organ during development. Formation of the murine placenta begins when the mesodermally-derived allantois (also a hematopoietic site in avian embryo) fuses with the ectoplacental cone at E8-8.5. Together with polar trophectoderm-derived giant cells, they form the chorionic disc. Thus, the fully developed placenta consists of the highly vascular labyrinth and the umbilical cord, both mesodermally-derived components (Caprioli et al., 1998; Cross, 1998; Downs, 2002).

The presence of hematopoietic cells (B-cell progenitors) in the placenta was first demonstrated in 1979 by Melchers (Melchers, 1979). More recently, the presence of *in vitro* clonogenic progenitors (HPP-high proliferative potential) in the placenta was demonstrated. Clonogenic hematopoietic progenitors from genetically marked (GPF) placentas were shown to give rise to large, multilineage colonies starting from E9 (20sp stage, before colonization of the fetal liver). Interestingly, progenitor frequency in the placenta was 2-4 times higher than observed in the yolk sac and fetal liver (Alvarez-Silva et al., 2003). Later in gestation, adult type of HSCs were found in the murine placenta. This has been demonstrated by *in vivo* transplantation studies (Ottersbach and Dzierzak, 2005; Gekas et al., 2005). The number of transplantable HSCs in the placenta has been shown to reach a peak at E12.5 and appear to decline after E13.5 (Gekas et al., 2005). This decline corresponds to the decline in B-progenitor potential found by Melchers (Melchers, 1979). Moreover, data obtained from placentas of Ly-6A GFP

transgenic embryos (where GFP was placed under control of *Ly-6A* regulatory elements; *section 1.5.1.*) showed not only that GFP is expressed in vascular labyrinth of the embryonic placenta of midgestation embryos, but also that beginning at E11 GFP marks all placental HSCs able to reconstitute the adult hematopoietic system (Ottersbach and Dzierzak, 2005). Thus, the placenta together with fetal liver are the tissues harboring the most abundant concentrations of HSCs during midgestation. It is not yet clear if the mouse placenta is a site of HSCs generation or is colonized by HSCs generated in other sites. It is also worthwhile to note here that it is as yet unknown, whether human placenta also functions as hematopoietic organ during development.

In addition to the yolk sac and placenta, hematopoietic activities have also been found in the major vessels that connect these tissues to the embryo body; the vitelline and umbilical arteries. Both of these vessels are only to some extent derived from extraembryonic mesoderm. The vitelline artery is formed at E8 as a truncal extension of the dorsal aorta branching off as the unpaired omphalomesentric artery at E10.5. The umbilical artery connecting dorsal aorta with the placenta appears one day later. It is generated by lengthening of the allantoic bud, following by vascularization (Garcia-Porrero et al., 1995; Downs et al., 2001).

Data described by de Bruijn et al. (2000) indicates that definitive HSC activity resides in the umbilical and vitelline arteries beginning from E10.5. Studies using the already mentioned Ly-6A GFP transgenic line, have shown that GFP expression was observed within cells of the hematopoietic clusters of vitelline and umbilical arteries in midgestation embryos (de Bruijn et al., 2002). Hence, HSC activity resides in the major vasculature of the mouse conceptus.

1.3.2. Intraembryonic hematopoietic sites

Hematopoiesis takes place not only in the extraembryonic sites, but also in the intraembryonic parts of the mouse embryo: aorta –gonad – mesonephros region (AGM), fetal liver and other secondary hematopoietic organs; i.e. spleen, and thymus, which are thought to be colonized by hematopoietic stem/progenitor cells produced in the PAS/AGM and the yolk sac (Medvinsky et al., 1993; Godin et al., 1993; Müller et al., 1994; Medvinsky and Dzierzak, 1996; Delassus and Cumano, 1996). It has been shown that AGM region is the first intraembryonic hematopoietic site, where at E10.5 adult repopulating HSCs are autonomously generated (Müller et al., 1994; Medvisky and Dzierzak, 1996). The mesodermally derived AGM region is localized between forelimbs and hind limbs of the midgestation embryo (E9.5-E12.5) (**Fig.3**). Other vertebrate species possess an analogous region to the murine AGM region. For example in *Xenopus* the equivalent is the dorsolateral plate (DLP) mesodermal tissue (Turpen and Knudson, 1982; Ciau-Uitz et al., 2000).

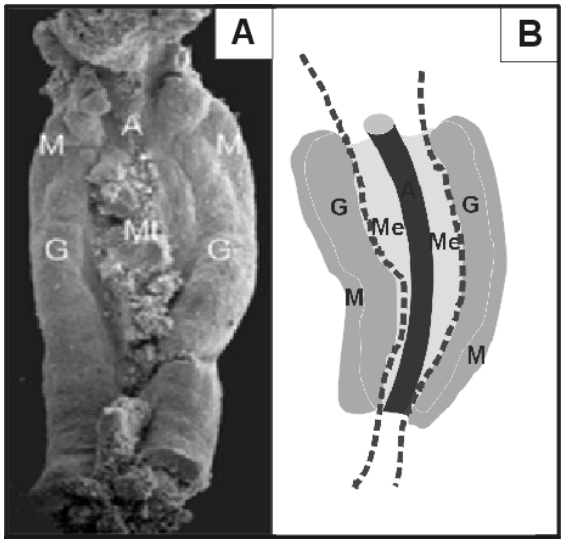

Figure 3. Anatomical characterization of the murine AGM region at E11.
The AGM region is one of the embryonic hematopoietic tissues. It is composed of the dorsal aorta (**A**) surrounded by mesenchyme (**Me**), and the lateral lying gonads (**G**) and pro/mesonephros (**M**). The paired G/M form the urogenital subregion. **A.** Electron micrograph of the E11 AGM region (de Bruijn et al., 2000a). **B.** Schematic subdissection of the E11 AGM, where dotted lines indicate separation of dorsal aorta and mesenchyme from the urogenital subregion (de Bruijn et al., 2000b).

In the early formation of the AGM region, the splanchnopleura forms from mesoderm and adjacent endoderm. At E8 paired aortae are generated from the mesodermal component, hence giving rise to the para-aortic splanchnopleura (P-Sp) (Godin et al., 1995). Afterwards at E8.5 these paired aortas are connecting via vitelline vessel to the YS. Subsequently, at E9 the aortae fuse to form the dorsal aorta, the major artery of the embryo body (Garcia-Porrero et al., 1995). One day later, at E10 the mesonephros and gonads start to develop and primordial germ cells migrate to the gonads. By E12 mesonephric regression occurs and the metanephros (definitive kidney) begins to develop (de Bruijn et al., 2000). After E12 hematopoietic activity in the AGM declines and the fetal liver becomes the main hematopoietic organ in the embryo. This sequence of events is similar in the human embryo and culminates in the fetal liver as the major hematopoietic site at 15 weeks of gestation. The fetal liver is derived from endoderm by E8.5. The hepatic rudiment starts to form as an evagination of the gut into septum transversum mesenchyme. Together, FGF and BMP signaling induce proliferation of the ventral foregut endoderm to form the liver bud (Zaret, 2001; Gualdi

et al. 1996, Chagraoui et al., 2003). Starting at E9.5 (28-32sp stage), the fetal liver anlagen is colonized by exogenous erythrocytes, hematopoietic progenitors and one day later by hematopoietic stem cells (Cudennec et al., 1981; Dzierzak and Medvinsky, 1995). The fetal liver remains hematopoietically active till the neonatal stage.

Subsequently to FL colonization, hematopoietic cells also colonize the spleen and thymic anlagen. The spleen begins to be colonized on E12.5 and harbors mainly multipotent hematopoietic cells (Godin and Cumano, 1999; Delassus and Cumano, 1996). The thymus is colonized between E12 and E13 and harbors mainly T-cell progenitors (Godin and Cumano, 1999). Later, at E15 hematopoietic multipotent cells seed also bone marrow, which two days later becomes a main site of B-lymphopoiesis (Delassus and Cumano, 1996). Shortly after birth the bone marrow becomes the predominant site of the definitive hematopoiesis (Dzierzak et al., 1998; Palis et al., 1999).

Importantly, the fetal liver, kidney, thymus and spleen are the secondary hematopoietic tissues during mouse development. While these secondary tissues are colonized by hematopoietic (stem) cells, it is generally thought that they are not sites of HSCs production (Godin and Cumano, 1996; Durand and Dzierzak, 2005). Instead, they function as specific hematopoietic environments allowing for the production of selected types of mature hematopoietic cells.

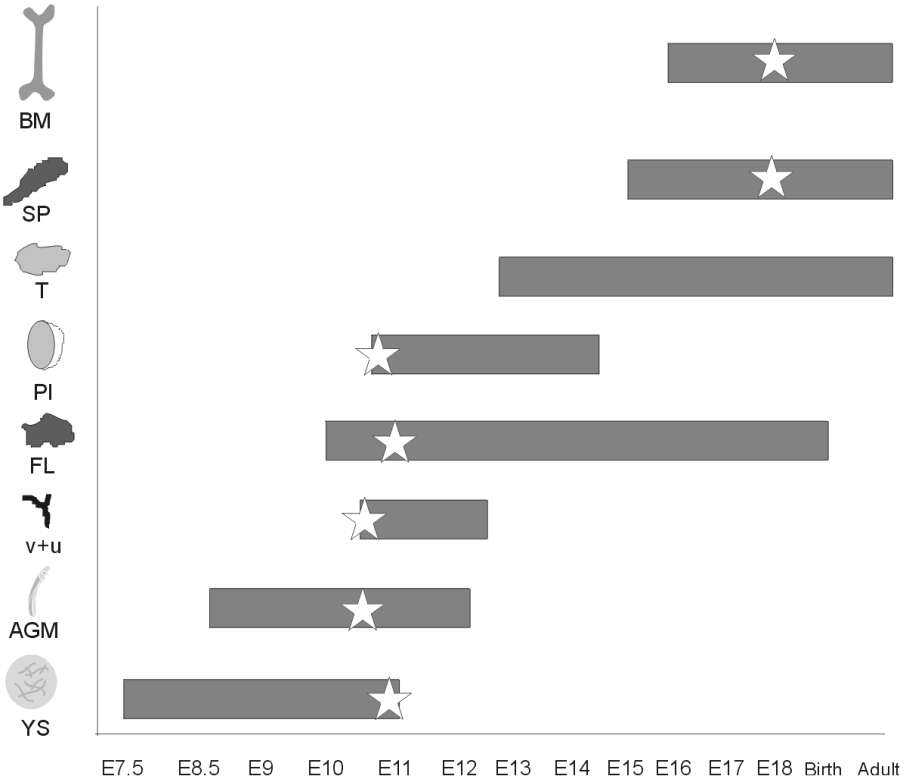

Figure 4. Hematopoietic (stem) cell distribution during mouse development.
The yolk sac is the first hematopoietic tissue, where hematopoietic cells arise starting from E7.5 (gray bar), and represents a site where primitive hematopoiesis takes place. The first adult repopulating HSCs appear at E10.5 in the AGM region (as indicated by the asterisk). At E11 definitive hematopoietic activity is found in the YS and FL. The vitelline and umbilical arteries and the placenta also harbor hematopoietic activity. Fetal thymus and spleen and subsequently bone marrow become organs seeded by hematopoietic cells, where further differentiation occurs. In adult the BM is the main site of hematopoiesis. Gray bars indicate the time when particular tissues harbor hematopoietic activity and asterisks show when HSC activity appears in these hematopoietic tissues.

1.4. Primitive versus definitive hematopoiesis

Hematopoiesis in the mouse takes place not only in different anatomical sites, but also at different developmental stages (**Fig.4**). To date, two types of hematopoiesis during ontogeny have been recognized; primitive and definitive. The term 'primitive' commonly used in association with yolk sac erythropoiesis is thought to represent transient populations of hematopoietic cells that are present only during the early stages of ontogeny, whereas the 'definitive' term defines the permanent, life-long adult type of hematopoiesis. Primitive hematopoiesis is initiated earlier than definitive and provides the first wave of hematopoietic cells within the mouse conceptus. Indeed, the first transient hematopoietic events start in the blood islands of the yolk sac, where between E7-7.5 (15-18 day in human) primitive (nucleated) erythrocytes are produced rapidly to allow the early developing embryo to grow and survive (Russel and Bernstein, 1966; Dzierzak et al. 1998; Dzierzak and Medvinsky, 1995). *In vitro* studies have demonstrated that at E7, the first detected hematopoietic progenitors consist of cells with primitive macrophage, granulocytic and erythroid potential. Such progenitors most likely give rise *in vivo* to already mentioned nucleated erythrocytes and primitive macrophages (Moore and Metcalf, 1970; Palis et al., 1999). Later at E8.25, another type of erythroid progenitor (burst-forming-unit-erythroid; BFU-E) can be detected in the yolk sac. The number of these progenitors increases proportionally to yolk sac cellularity between E8-9 (Palis et al., 1999). At this developmental stage, besides the presence of erythroid progenitors (BFU-E), multipotent and lymphoid progenitors representing definitive progenitors were also detected in the yolk sac (Cumano et al., 1996; Palis and Yoder, 2001). Simultaneous detection of lymphoid progenitors in the YS and in the embryo at E8.5 indicates that these cells are already in the circulation at this early embryonic stage (Godin et al., 1995). The presence of such definitive progenitors in the YS only after the establishment of the circulation at E8.5, suggests that they are not generated in the yolk sac and that their maturation occurs in other hematopoietic tissues (Robertson et al., 1999). At late E9 the first *in vivo* definitive multipotent progenitors (colony-forming-unit-spleen; CFU-S) appear in the yolk sac

(Medvinsky et al., 1993; Dzierzak and Medivsky, 1995 review), but no adult repopulating hematopoietic stem cell activity could be detected in the yolk sac until E11 (Müller et al., 1994; Medvinsky and Dzierzak, 1996).

The presence of both primitive and definitive erythroid progenitors in the YS brought additional interest in careful examination of early hematopoietic commitment stages to identify precursors of erythroid lineages. Using the ES (embryonic stem cell) systems, contradictive data from different sources claimed either the existence a common precursor for primitive and definitive erythroid lineage (Kennedy et al., 1997) or independent origin for these both lineages (Nakano et al., 1996; Fujimoto et al., 2001). Additionally, while the primitive (yolk sac) hematopoiesis ends at E13, when the yolk sac degenerates, the production of hematopoietic cells in definitive hematopoiesis is maintained during the entire mouse lifespan.

Definitive hematopoiesis differs from the yolk sac primitive hematopoiesis because it involves the production of all different types of blood cells (erythroid, myeloid, lymphoid and megakaryocytic cells) (Metcalf and Moore, 1971). Moreover, in contrast to the yolk sac erythropoietic cells, definitive erythrocytes are enucleated and expressing adult globin chains (α, βmajor) (Shivdasani and Orkin, 1996; Palis et al., 1999). Thus, different but somewhat overlapping molecular programs are thought to direct the maturation of the primitive and definitive lineages. Definitive hematopoiesis occurs throughout development predominantly in P-Sp/AGM, FL, placenta and the adult bone marrow (BM). At midgestation the main intraembryonic site of hematopoietic cell generation is the P-Sp/AGM, where by E9.5 multipotent progenitors (CFU-S_8; *section 1.5.2.*) are detected (Medvinsky et al., 1993). Later, at E10.5 first adult type of HSCs are produced autonomously in this region (Müller et al., 1994; Medvinsky and Dzierzak, 1996). Between E10.5-E11.5 a second wave of definitive multipotent hematopoietic progenitors (CFU-S_{12}) and HSCs, coming predominantly from AGM, start to colonize the fetal liver (Medvinsky et al., 1993). Interestingly, a potential role of the embryonic placenta in definitive hematopoiesis was also reported (Ottersbach and Dzierzak, 2005; Gekas et al., 2005). In the embryonic placenta the first adult HSC activity is detected between E10.5-E11 (Ottersbach and Dzierzak, 2005; Gekas et al., 2005). These HSCs may also migrate and colonize the YS. This is suggested by the quantitative analysis of HSC activity in the midgestation mouse embryo (Kumaravelu et al., 2002) and the fact that the number of HSCs in the FL cannot be accounted for solely on the basis of HSCs generation in the AGM.

The main site of maintenance and expansion of definitive HSCs during the ontogeny is the fetal liver. Fetal liver hematopoiesis initiates around E10.5-E11 (day 35-42 in humans) in the mouse, although the definitive erythropoiesis begins already at E9 (28-32sp), when the first wave of hematopoietic progenitors from YS colonize the fetal liver (Chagraoui et al., 2003; Shivdasani and Orkin, 1996; Palis et al., 1999; Cudennec et al., 1981). HSCs are present in the FL from E11 until birth when the BM becomes the main site of definitive hematopoiesis (Dzierzak et al., 1998).

1.5. Phenotypic and functional characterization of hematopoietic cells

1.5.1. Identification, isolation and characterization of hematopoietic progenitor/stem cells

Hematopoietic stem cells are found in the mouse adult BM at very low frequencies (1 to 10 in 10^5 nucleated cells) (Boggs et al., 1982). This very low frequency of HSCs and the important function of HSCs in the clinical transplantations have encouraged researchers to develop methods for HSCs isolation and expansion. The absence of an exclusive marker to define HSCs has led to the development of a diverse number of isolation procedures for hematopoietic progenitor/stem cells enrichment from a heterogeneous cell population.

A routinely used method for HSC enrichment is fractionation by a density gradient (Visser et al., 1984). This method is based on differences in density of different types of hematopoietic cells, including stem cells. The density gradient, created by centrifugation of cells in media such as Percole, Ficoll or Hypaque, allows for 2-5-fold HSC enrichment. However this fractionation method has some disadvantages. It is difficult to separate lymphocytes from HSCs, because their densities overlap. Thus, the HSC enriched fraction is still contaminated by lymphocytes. The other drawback of this density gradient centrifugation is that it removes some light density cells (typically 1-30%), where HSC activity has been found (Wognum et al., 2003). This means that a part of the HSC population will be lost.

Isolation of HSCs can also be performed based on differential efflux of some fluorescent dyes such as Rhodamine 123 and Hoechst 33342. Hoechst 33342 and Rhodamine 123 act through ABC transporters, which are differentially expressed by HSCs relative to other cell types (Bertolini et al., 1997; Goodell et al., 1996; Uchida et al., 2004). HSCs stained with Hoechst are found in the side population (SP), a population with a specific graphical location in flow cytometry analysis (Goodell et al., 1996). Analysis of HSC activity in *in vitro* and *in vivo* assays of the Rhodamine 123 and Hoechst 33342 stained fractions of the FL and adult BM cells revealed extremely variable efflux activities of HSCs according to their developmental and activation state. Reduced percentages of embryonic HSCs able to efflux the Hoechst efficiently and failure in Rhodamine 123 efflux was observed as compared to adult HCSs. For most of adult BM HSCs the efflux abilities of both dyes were retained until 4 weeks of age in mouse, but by 8 weeks of age the abilities were reversed (Uchida et al., 2004). Moreover, *in vitro* culture with cytokines and *in vivo* treatment with toxins such as 5-fluorouracil (5-FU), which kills all actively dividing cells, caused variable efflux abilities of the HSCs (Cooper and Spangrude, 1999; Uchida et al., 2004). Thus, reliability of HSC isolation by the efflux potential of these two dyes depends on the age and the state of activation of HSCs.

The most frequently used enrichment methods for HSCs are based on cell surface protein expression. Different types of hematopoietic cells vary in the array of

proteins expressed on their cell-surface. Through the application of a variety of fluorochrome-coupled antibodies specific for these proteins, distinct hematopoietic cell populations can be visualized, detected and sorted. The development of FACS technology became a very powerful method, because these different populations, containing hematopoietic progenitor/stem cells can be isolated separately (Spangrude, 1989). An easy way to separate differentiated cells from hematopoietic progenitors/stem cells is to apply the cocktail of antibodies specific for hematopoietic differentiation lineage markers (B-cell specific anti-B220; T-cell specific anti-CD4 and anti-CD8 or anti-CD3, erythroid specific anti-Ter119, granulocyte specific anti-Gr1). This negative selection method enriches for hematopoietic progenitor/stem cells around 10-fold (Spangrude et al., 1998; Cooper and Spangrude, 1999) and this population is commonly known as lineage negative (Lin^-). In the isolation of HSCs this negative selection is followed by a second step, a positive selection for different combinations of markers, which are expressed on hematopoietic progenitors or HSCs. To isolate highly HSC enriched populations some of the common combinations are c-kit^{high} Thy1.1^{low} $Lin^{-/low}$ Sca-1^+, Thy1.1^{low} Lin^- Sca-1^+ Rhodamine123^{low} (Morrison et al., 1995), or $CD34^{low/-}$ c-kit $^+$ Sca-1^+ Lin^- (Osawa et al., 1996). However the expression of these HSC markers, similar to dyes efflux, changes depending on the developmental source of HSC or activation state of HSC influenced by *in vivo* or *in vitro* treatments (Taijma et al., 2000; Uchida et al., 2004). The cell-surface molecules used for negative and positive selection are summarized in **Table 1** and brief descriptions of some of these molecules are provided here.

One of the markers used for positive HSC enrichment is CD34, a sialomucyn glycoprotein. It has been shown to be a reliable marker for HSCs during mouse embryonic development (Donnelly et al., 1999; Morel et al., 1998). However the fraction of adult BM HSCs highly enriched for HSCs was shown to be CD34 $^{-/low}$ (Taijma et al., 2000).

A controversial HSC marker is CD41 (Mitjavila-Garcia et al., 2002; Mikkola et al., 2003). CD41 is a cell adhesion receptor expressed during development at variable levels in different hematopoietic tissues. It may have a function as an adhesion molecule in HSC migration. CD41 is also found on megakaryocytes and platelets. Based on *in vivo* assays most HSC activity was found in sorted CD41 negative population of BM, whereas for some embryonic tissues such as fetal liver hematopoietic activity was detected both in CD41 positive and negative subset (Mitjavila-Garcia et al., 2002). However, another report has shown that BM HSCs are $CD41^+$ c-kit^+ (Corbel and Salaun, 2002). Thus, it still remains unclear if CD41 is a potential marker for definitive HSC isolation. Some additional *in vivo* assays have shown that HSC activity could be found in the $CD41^-$ fraction in the AGM region (unpublished data, Dzierzak lab).

One of the most interesting and often used markers for enrichment of embryonic and adult HSCs is Sca-1 (known as stem cell antigen-1 or as lymphocyte activation protein-6A (Ly-6A/E)) marker. Sca-1 belongs to Ly-6 superfamily of phosphatidylinositol (GPI) linked cell-surface glycoproteins (Gumly et al., 1992; Ito

Table 1. The murine hematopoietic (stem) cell markers (updated and adapted from *PhD* thesis of C. Orelio).

Marker / CD number		Marker type	Tissue/cell type expression	References
c-kit	CD117	tyrosine kinase receptor (receptor for stem cell factor-SCF)	HSC hematopoietic progenitors vascular endothelium testis melanocytes, mast cells	Ashman et al., 1999 Kawashima et al., 1996
αGPIIb integrin	CD41	cell adhesion receptor	HSC hematopoietic progenitors vascular endothelium megakaryocytes, platelets	Ferkowicz et al., 2003 Mikkola et al., 2003 Mitjavila-Garcia et al., 2002
Sca-1 (Ly6A/E antigen)		transmembrane glycoprotein	HSC hematopoietic progenitors lymphoid cells vascular endothelium, brain	Bradfute et al., 2005 Ma et al., 2002a Ma et al., 2002b Miles et al., 1997
	CD45	pan leucocyte antigen; transmembrane glycoprotein	HSC hematopoietic cells excluding mature red blood cells	Bertrand et al., 2005 McKinney-Freeman et al., 2002 Mikkola et al., 2003
	CD 34	sialomucin glycoprotein	hematopoietic progenitors vascular endothelium embryonic fibroblasts	Morel et al., 1998 Osawa et al., 1996 Sato et al., 1999
Flk-1		tyrosine kinase receptor	HSC vascular endothelium	Kabrun et al., 1997 Yamaguchi et al., 1993
Mac-1 αM β2 integrin	CD11b	cell adhesion receptor	HSC macrophages lymphoid cells	Sanchez et al., 1996 Corbi et al., 1988 Springer, 1990
PECAM-1	CD31	platelet endothelial cell adhesion molecule -1	HSC hematopoietic progenitors vascular endothelium lymphoid and myeloid cells	Li et al., 2005 Bertrand et al., 2005 Ottersbach and Dzierzak, 2005
Tie-2 /Tek		tyrosine kinase receptor	HSC endothelial progenitors vascular endothelium	Hamaguchi et al., 1999 Hsu et al., 2000 Takakura et al., 1998
AA4.1		cell adhesion transmembrane protein	HSC hematopoietic progenitors vascular endothelium lungs, heart, bone marrow	Bertrand et al., 2005 Godin et al., 1999 Kim et al., 2004 Petrenko et al., 1999
EPCR	CD201	endothelial protein C receptor	HSC vascular endothelium embryonic placenta	Balazs and Mulligan, 2003 Fukudome and Esmon, 1995 Ivanova et al., 2002
endoglin	CD105	transmembrane accessory receptor for TGF	HSC vascular endothelium full-term placenta stromal cells HSC	Chen et al., 2002 Chen et al., 2003 Cheifetz et al., 1992 Gougos et al., 1990
FGFR-1		fibroblast growth factor receptor	skin, hepatocytes developing brain motor neurons	de Haan et al., 2003

et al., 2003). It is thought that Sca-1 plays a minor role in adult murine hematopoiesis

since Ly-6A deficient mice showed mild thrombocytopenia, a decrease of hematopoietic progenitors in BM and impaired self-renewal of BM HSCs (Ito et al., 2003; Bradfute et al., 2005). However, the function of Sca-1 in embryonic hematopoiesis still remains unclear. Expression of Sca-1 is not restricted to hematopoietic cells but is also observed in other tissues such as vascular endothelium, kidney, brain, liver and epithelium (Brakenhoff et al., 1995; Miles et al., 1997; Ma et al., 2002a). One of the disadvantages in using Sca-1 as a HSC marker is the different mouse strain-specific alleles of the Ly-6 superfamily. The Ly-6A and E alleles are expressed differentially on HSCs (Gumly et al., 1995). Only 25% of adult BM HSCs express Sca-1 in the Ly-6E allelic strain, whereas in the Ly-6A strain almost all (99%) of adult HSCs are within Sca-1 positive fraction (Spangrude and Brooks, 1993; Miles et al., 1997). In the term of enrichment, using a monoclonal antibody specific for the Sca-1 epitope expressed by both A and E alleles, adult BM HSCs can be enriched around 100-fold (Ma et al., 2002a; Ma et al., 2002b). In addition, a Sca-1 GFP transgenic mouse line has been generated and used for enrichment of HSCs without the need of antibodies. The GFP transgene has been shown to label all HSCs throughout all developmental stages and appeared to be an important easily visualized marker for the enrichment and localization of the first HSCs emerging in the mouse embryo (Ma et al., 2002a; de Bruijn et al., 2002).

Another commonly used marker for HSC isolation is c-kit, a tyrosine kinase receptor. c-kit together with its ligand SCF (stem cell factor) are important factors for proper functioning of HSCs and hematopoietic progenitors (McCulloch et al., 1964; Capel et al., 1989; Ashman, 1999; Waskow et al., 2002). Indeed, for many years it was known that the W/W natural mouse mutants, carrying mutation in the c-kit receptor, show a hematopoietic phenotype such as macrocytic anemia leading in some severe cases to embryonic lethality (Geissler et al., 1988; Chabot et al., 1988; Nocka et al., 1990). c-kit is expressed on embryonic and adult hematopoietic progenitors/ stem cells (Ikuta and Weissman, 1992; Sanchez et al., 1996; Dagher et al., 1998). In the adult BM 5-10% of cells express c-kit (Ikuta and Weissman, 1992). It has been shown that transplantation of Lin^{-} $Sca\text{-}1^{+}c\text{-}kit^{+}$ $Thy1.1^{+}$ subset results in the long-term repopulation of lethally irradiated adult recipients (Spangrude et al., 1988). Moreover, the data of Ikuta and Weissman show that whereas Lin^{-} $Sca\text{-}1^{+}Thy1.1^{low}$ $c\text{-}kit^{+}$ fraction is highly enriched in LTR-HSC and progenitor activities, in the Lin^{-} $Sca\text{-}1^{+}$ $Thy1.1^{low}$ $c\text{-}kit^{-}$ subset only progenitor (CFU-S) activity remained (Ikuta and Weissman, 1992).

Interestingly, within the already HSC enriched populations it is also possible to isolate the most primitive and extensively self-renewing long-term (LTR) and restricted self-renewing short-term (STR) subpopulations of HSCs. Absence of Flk-2 marker within the subset of cells with phenotype: $c\text{-}kit^{+low}Thy1.1^{low}Lin^{-/low}Sca\text{-}1^{+}$ distinguish the LTR-HSCs from STR- HSCs, which express Flk-2 and give rise to multipotent progenitors ($c\text{-}kit^{+/low}Thy1.1^{-}Lin^{-/low}Sca\text{-}1^{+}Flk\text{-}2^{+}$) without any self-renewal potential (Christensen and Weissman, 2001).

Finally, the application of cytotoxic drugs (like 5-FU or hydroxyurea) or alkylating agents (derivatives of cyclophosphamide) have been used for *in vivo* HSC enrichment. It is known that in contrast to hematopoietic progenitors, quiescent adult HSCs are resistant to 5-FU. *In vivo* 5-FU-treatment ablates only actively dividing cells, thus increasing the frequency of BM HSCs by 5-10 fold (Palis et al., 2001; Lemieux and Eaves, 1996). Cyclophoshamide is used clinically to destroy malignant cells in patients before transplantation and to induce HSC mobilization (Szumilas et al., 2005; Lapidot et al., 2005). It is known that primitive hematopoietic cells (including HSCs) are resistant to the alkylating agents, because they produce high level of aldehyde dehydrogenase (ALDH), which inactivates the agents (Sahovic et al., 1998; Jones et al., 1995). Fluorescent-labelled ALDH substrates are being used for the isolation of mouse or human adult HSCs by FACS (Jones et al., 1995; Storms et al., 1999).

1.5.2. Assays to identify hematopoietic progenitors and stem cells

The criteria by which hematopoietic cells are identified are based on a wide variety of functional assays summarized in **Figure 5**. These assays allow an appropriate positioning of the tested hematopoietic cells within the differentiation hierarchy based on their proliferative and differentiation potential.

The first hematopoietic assays were developed in the 1960's to quantitatively and qualitatively test hematopoietic progenitor activity in a variety of tested cell populations (Till and McCulloch, 1961; Bradley and Metcalf, 1966). One of these assays, CFU-S (colony forming unit - spleen) *in vivo* assay relies on formation of macroscopic colonies of myeloid and erythroid lineages. At 8-12 days following the intravenous injection of BM cells into lethally irradiated adult mice these colonies are visible as white or red nodules on the spleen (Siminovitch et al., 1963; Curry and Trentin, 1967; Moore and Metcalf, 1970). CFU-S colonies formed 12 days after injection result from more immature progenitors than the colonies formed at 8 days after injection. Only some of these colonies are able to self-renew, but nevertheless, none of them possess long-term repopulating activity characteristic for the hematopoietic stem cells. It is assumed that obtained number of CFU-S colonies corresponds to the number of myeloid/erythroid progenitors in the injected input population (Ploemacher and Brons, 1988; Medvinsky et al., 1993). Other colony assays to test activities of the thymic progenitors and B-lymphoid precursors are the thymic colony-forming assay (CFU-T) and Whitlock-Witte bone marrow culture, respectively (Spangrude et al., 1991; Muller-Sieburg et al., 1986).

Despite of the wide use of *in vivo* progenitor assays, more direct *in vitro* assays were developed to test for mature hematopoietic progenitors (Metcalf and Nicola, 1984; Metcalf, 1984). The hematopoietic potential of test populations of cells is determined after culture for several days in semisolid medium supplemented with conditioned medium containing growth factors. These first colony forming assays have been improved significantly over the years and nowadays there are different commercially

available methylcellulose media with well defined conditions and recombinant hematopoietic factors. The most widely used *in vitro* hematopoietic progenitor assay is the CFU-C (the colony forming unit-culture) assay. In this assay, the test population of hematopoietic cells is plated in methylcellulose in the presence of defined set of cytokines. After 7-14 days of the culture the erythroid/myeloid colonies are scored (Metcalf, 1984).

With regard to the testing of more primitive hematopoietic activity, a number of *in vitro* assays have been developed. The most commonly used assays are LTC-IC (the long-term culture-initiating cell) (Sutherland et al., 1990; Miller and Eaves, 1997) and CAFC (cobblestone-area-forming cell) (Ploemacher, 1991; Ploemacher et al., 1997). In the LTC-IC assay, a cell population enriched for HSCs is co-cultured with supportive stromal cell layer (Sutherland et al., 1990; Miller and Eaves, 1997). After long-term co-culture, hematopoietic cells are harvested and tested for CFU-Cs to determine if the culture contained true multipotent hematopoietic cells that suggest the presence of self-renewing HSCs. The stromal cells in general are an important component of the *in vivo* microenvironment, where hematopoietic (stem) cells reside. Similarly, in the CAFC assay the hematopoietic cells are co-cultured in the presence of stromal cell layer. HSCs and hematopoietic progenitors are plated in a serial dilution manner and scored for so-called cobblestone areas between 7 and 35 days of the culture. Whereas the cobblestones areas arising early in the culture represent more mature hematopoietic progenitors, the cobblestones at day 35 are considered to be the most primitive hematopoietic cells, including the HSCs (Ploemacher, 1997). In both types of these *in vitro* systems the stromal layer induces the expansion of the hematopoietic progenitor/stem cells. Nevertheless, whether the progenitor/stem cells are functional *in vivo* cannot be addressed by these assays. For the study of small numbers of developing hematopoietic progenitor/stem cells in the mouse embryo, Godin et al. developed the single cell multipotential cell assay (SMA). This is a two-step *in vitro* assay, where first step includes a supportive S17 stromal monolayer, which allows for clonal expansion of the uncommitted progenitors and, after 10-15 days, the second step allows for the progeny derived from each single hematopoietic cell to be tested for myeloid/lymphoid lineage activity in differentiation assays (Godin et al., 1995).

Apart from all the *in vitro* hematopoietic assays, the most stringent assay to test the functionality of HSC is the long term *in vivo* transplantation assay, which results in the permanent, multilineage, high level reconstitution of the hematopoietic system. Unsorted cells or enriched in HSC cell populations are injected into lethally irradiated recipients ablated of endogenous hematopoiesis (Yoder et al., 1996; Dzierzak et al., 1998; Dzierzak and Medvinsky, 1995). Transplantation can be performed in a direct or competitive manner, where in the latter the marked donor HSCs are titrated and coinjected with a known number of autologous cells. Although irradiation of recipients is the most common way to ablate endogenous hematopoietic system, busulfan was used for myeloablative preconditioning before injection in neonatal recipient mice (Yoder and Hiatt, 1997).

An important factor in the design of a transplantation experiment is that the injected fraction of cells has to differ from the recipient cells. This difference can be obtained in several ways such as by allelic hematopoietic markers (Thy1.1 or CD45.1/CD45.2) (Spangrude et al., 1988), enzymes isoforms (PGK or GPI; Yoder et al., 1996), hemoglobin polymorphisms (Yoder and Hiatt, 1997), sex chromosome (Y which can detect male cells in a female recipient) or use of genetic markers such as a transgene (Müller et al., 1994). From all these mentioned markers, which allow distinguishing between donor- and recipient-derived cells, the CD45 allelic marker, expressed on all hematopoietic cells excluding red blood cells and platelets, carries some advantages. It can be easily visualized by monoclonal antibodies, followed by flow cytometry analysis and in some genetic backgrounds of mouse strains is the most suitable to distinguish the hematopoietic cells between the donor and recipient (Trowbridge and Thomas, 1994; Molecular Hematology, eds. Provan and Gribben, 2005). However, because the CD45.1/CD45.2 system is based on the congenic C57BL/6 and BL6-SJL strains, it is sometimes difficult to study HSCs in mice with particular genetic backgrounds. In such cases a PCR based method detecting a transgene or gene targeting marker in the DNA of the transplant recipient or usage of different allelic hemoglobins or enzyme isoforms is highly advantageous.

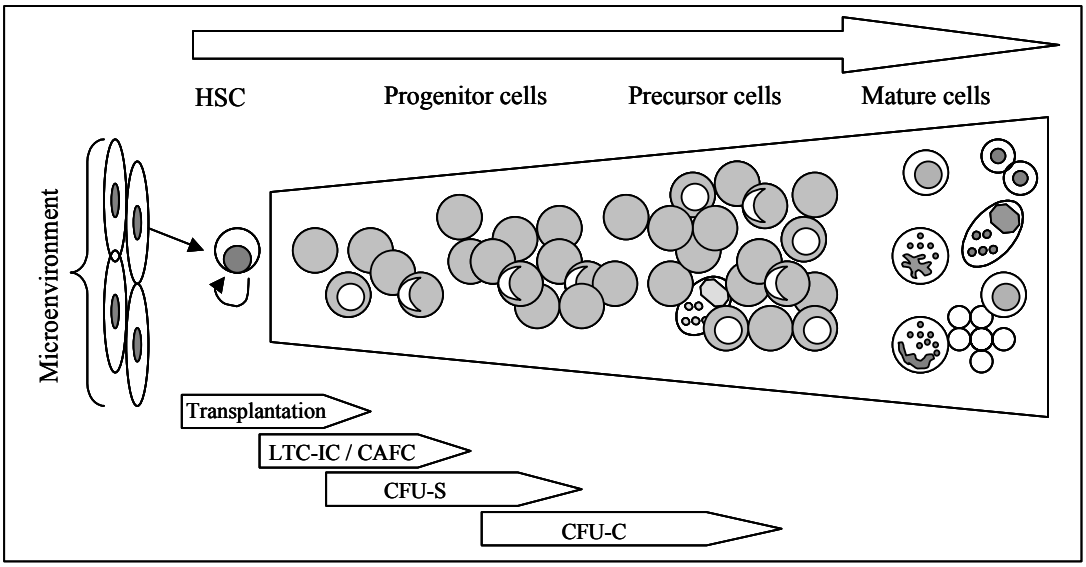

Figure 5. Overview of *in vivo* and *in vitro* assays to test different hematopoietic activities. Stem cell activity can be assayed *in vivo* by a transplantation assay; multipotent progenitors are tested *in vivo* by CFU-S assay and *in vitro* by LTC-IC and CAFC using co-culture systems. The CFU-C assay tests more committed progenitors. HSC- hematopoietic stem cell; LTC-IC (the long-term culture-initiating cell); CAFC (cobblestone-area-forming cell); CFU-S (colony forming unit - spleen); CFU-C (the colony forming unit-culture) (adapted from Fig.3.2 in Molecular Hematology, 2nd edition, eds. Provan and Gribben, 2005).

1.5.3. Phenotypic characterization of embryonic and adult HSCs

HSCs in the embryo are functionally divided into two types, immature and mature HSCs. These HSCs are defined by their ability to repopulate recipients in a transplantation scenario. Mature HSCs are able to repopulate irradiated adult recipient mice, whereas immature HSCs cannot. Instead these HSCs repopulate hematopoietic compromised neonatal recipients (Dzierzak et al., 1998). As compared to the mature or adult type of HSCs, neonatal repopulating HSCs appear to have restricted proliferative potential and/or bone marrow homing potential (Yoder et al., 1997a; Yoder et al., 1997b). The first mature adult repopulating HSCs are found in the AGM at E10.5. Mature HSCs are also found in the vitelline and umbilical arteries and the placenta and one day later these cells are also detected in the yolk sac and FL (Müller et al., 1994; Medvinsky and Dzierzak, 1996; Huang and Auerbach, 1993; Ottersbach and Dzierzak, 2005; Gekas et al., 2005).

As previously mentioned, mature adult repopulating HSCs in the yolk sac were not found before E11. However, Yoder and colleagues have shown that at E9 the YS contains neonatal repopulating HSCs (Yoder et al., 1997a; Yoder et al., 1997b). Moreover, others have found multipotential progenitors in the P-Sp before the presence of mature HSCs. These progenitors can repopulate immune deficient (Rag2-/-: γc-/-) adult recipients (Cumano et al., 2001). However, this repopulation is at low level. Thus, it is thought that these early cells represent an immature HSCs population (Cumano et al., 2001; Godin et al., 1995; Cumano et al., 1996).

Both neonatal repopulating HSCs from yolk sac and adult repopulating E11 AGM HSCs are c-kit$^+$CD34$^+$ (Sanchez et al., 1996; Yoder et al., 1997a). Approximately 50% of AGM HSCs are also Sca-1$^+$ and Mac-1$^+$. The fact that both neonatal repopulating HSCs and mature HSCs express similar markers (c-kit and CD34) (Sanchez et al., 1996; Yoder et al., 1997a) suggests that the immature neonatal HSCs found earlier in murine hematopoietic ontogeny might be direct precursors of definitive HSC, the so called "pre-HSC" (Dzierzak et al., 1998). They have been found to express AA4.1 marker, expressed also on FL HSCs (Godin et al., 1995; Jordan et al., 1990). Thus, a panel of phenotypic characteristics should allow at least for some understanding of the possible lineage relationships in the embryo between immature HSCs and mature HSCs.

With regard to mature HSCs from E11YS, they express the AA4.1 and Sca-1 markers, which are also expressed by FL HSCs (Huang and Auerbach, 1993; Jordan et al., 1990). FL HSCs also express c-kit, CD34, and Thy1 (Müller et al., 1994; Sanchez et al. 1996). And similar to AGM HSCs, E13/14FL HSCs have been shown to express Mac-1 (Morrison et al., 1995).

Recently adult type of HSCs were found in embryonic placenta. Using the Ly-6A (Sca-1) GFP transgenic mice it has been shown that beginning at E11, all placental adult repopulating HSCs are GFP$^+$ and that GFP expressing cells in the placental labyrinth coexpress other HSC markers, such as CD34 and CD31 (Ottersbach and

Dzierzak, 2005). In addition, Gekas et al., showed by phenotypic and functional analysis that all E12.5 HSCs in the placenta are in the c-kit$^+$CD34$^+$ subset of cells. Moreover some CFU-S activity (13%) was found in c-kit$^+$CD34$^-$ population (Gekas et al., 2005).

Contrary to AGM and placenta HSCs, most of adult BM HSCs are considered to be CD34$^-$ (Matsuoka et al., 2001), although CD34 expression was reported as reversible since BM HSCs activated *in vivo* (5-FU treatment) or *in vitro* became CD34$^+$. Following transplantation they again lost CD34 expression (Sato et al., 1999; Ogawa et al., 2001). Further analysis of different murine developmental stages with regard to CD34 expression, revealed that all adult BM HSCs from perinatal to 5-week-old mice are CD34$^+$, whereas in 2 weeks older mice CD34$^-$ HSCs are appearing and in much older animals (10-20 weeks old) the majority of the HSCs do not express CD34 (20% of the adult stem cells expressed CD34) (Ogawa et al., 2001).

Additionally, expression of Mac-1 and other markers such as AA4.1 and CD45 distinguish FL HSC cell-surface phenotype from adult BM HSC phenotype, since BM HSC activity reside in populations negative for Mac-1$^-$, CD45$^-$, and positive or negative for AA4.1 (Jordan et al., 1990; Rebel et al., 1996; Orlic et al., 1993). Nevertheless, HSCs from both FL and adult BM share some cell-surface phenotype such as Thy1^{low}Lin $^-$Sca-1$^+$ (Spangrude et al., 1991). Finally, studies on Ly-6A (Sca-1) GFP reporter mice revealed that similar to HSCs in the embryo, all adult BM HSCs are in the GFP$^+$ fraction (Ma et al., 2002a).

1.6. Developmental signaling pathways involved in hematopoiesis and the microenvironment

During mouse development hematopoietic cells interact with the surrounding microenvironment. The microenvironment is an essential component of hematopoietic niche, which regulates hematopoietic cellular behavior, including proliferation, differentiation and apoptosis. The regulatory signals from the microenvironment are restricted not only to direct cell-cell contact (for example between hematopoietic and stromal cell), but also involve soluble molecules such as cytokines (interleukins or growth factors) and signaling factors such as TGFβ (transforming growth factor β), Wnt, Notch and Hedgehog. Some of these molecules, especially the latter ones have been implicated during mouse development in the induction and regulation of different types of hematopoietic cells by changing gene expression in target cells. The role of some important signaling pathways involved in mouse developmental hematopoiesis will be shortly discussed here.

TGFβ signaling

Both the hematopoietic and the vascular system are derived from mesoderm. One of the signaling pathways involved in mesoderm formation is TGFβ signaling.

TGFβ is a member of the TGFβ superfamily, which also includes activins and BMPs (bone morphogenetic proteins). These factors regulate various cellular processes such as proliferation, differentiation, migration and cell fate decisions (Chen et al., 1996; Jonk et al., 1998; Itoh et al., 2000). The mechanism of TGFβ signaling is shared between all the family members and is initiated by binding of the ligand to specific receptors and thereby activating intracellular signal transmitters, such as Smad proteins (Jayaraman and Massague, 2000; Itoh et al., 2000; Quinn et al., 2001).

Several studies have shown that members of the TGFβ superfamily play important role in blood formation in the embryo and in regulation of hematopoietic cells in the adult. Knockout studies in mice showed that BMP-4-deficient embryos have impaired mesoderm formation and reduced erythropoiesis in the yolk sac and the embryo (Winnier et al., 1995). Interestingly, BMP-4 expression has been observed in the ventral part of dorsal aorta in the human AGM region, suggesting a role for BMP-4 in AGM hematopoiesis (Marshall et al., 1999; Garcia-Porrero et al., 1995). Moreover, addition of BMP-4 to *in vitro* culture of the murine ES cells leads to the increase number of the hematopoietic cells (Johansson et al., 1995).

With regard to another member of TGFβ superfamily, it has been shown that absence of the TGFβ-1 results in impaired formation of vasculature and yolk sac hematopoiesis (Dickson et al., 1995). In the adult, TGFβ-1 is well known for its *in vitro* inhibitory effect on the proliferation of hematopoietic progenitor/stem cells and also for its effects on their differentiation (Keller et al., 1990; Sitnicka et al., 1996). However, conditional inactivation of the specific TGFβ receptor in adult mice showed that TGFβ signaling *in vivo* is not crucial for maintaining the quiescent state of HSCs and their pool in the adult bone marrow (Larsson et al., 2005).

Wnt signaling

The Wnt signaling pathway is conserved in evolution from Drosophila to human. The Wnt family consists of secreted glycoproteins (Cadigan and Nusse, 1997), which bind to a transmembrane receptor called Frizzled (Wodarz et al., 1998; Tamai et al., 2000; Pinson et al., 2000; Reya and Clevers, 2005). In the so-called "canonical" Wnt signaling pathway, the key player is β-catenin. In the absence of receptor activation, β-catenin is phosphorylated and thereby targeted for degradation. Activation of Wnt receptors inhibits β-catenin degradation and leads to accumulation of β-catenin in the nucleus and enhanced binding to LEF-TCF transcription factors. These β-catenin/TCF complexes induce activation of target genes through binding to specific DNA sequences (Fearon and Cadigan, 2005; Duncan et al., 2005). To note, a β-catenin-independent signaling was also described (Peifer and Polakis, 2000).

In the embryo, expression of several Wnt members was detected in different hematopoietic tissues such as the yolk sac, AGM and fetal liver (Austin et al., 1997; Orelio and Dzierzak, 2003). Wnt5a and Wnt10b were shown to stimulate the expansion of fetal liver hematopoietic progenitors *in vitro* (Austin et al., 1997). However, there is

still no direct evidence that Wnt5 is involved in regulation of hematopoiesis in the embryo, since Wnt5a-deficient embryos do not display a hematopoietic phenotype (Yamaguchi et al., 1999). Interestingly, several Wnt related transcripts were found to be expressed in the AGM between E10 and E12, at the time when HSC activity can be detected in this region. Furthermore, expression of Wnt3 was observed near the dorsal aorta in the E12 AGM by immunohistochemistry (C.Orelio, unpublished data).

In the adult, Wnt signaling has also been implicated in regulation of HSC activity. Overexpression of axin (a negative regulator of Wnt signaling) in murine BM HSCs resulted in reduced repopulating potential of the HSCs (Reya et al., 2003). Moreover, Wnt3a has been shown to induce an expansion of HSCs in *in vitro* cultures (Reya et al., 2003). All these data together reveal an important regulatory role for Wnt signaling in developmental hematopoiesis.

In addition, integrated Wnt and Notch signaling has been shown *in vitro* to play important role in maintenance of undifferentiated HSCs (Duncan et al., 2005), suggesting that also the Notch pathway is implicated in hematopoiesis. Hence, Wnt signaling may play, along with other signaling pathways, a supporting role in development of the murine hematopoietic system.

Notch signaling

The Notch signaling pathway is also highly conserved throughout evolution and plays a crucial role in the specification of cell fates. Notch signaling often acts by inhibiting differentiation along a particular pathway while allowing or promoting self-renewal or differentiation along alternative pathways. Disruption or malfunction of the Notch signaling leads to very severe abnormalities during development such as impaired neurogenesis, somatogenesis, angiogenesis (Artavanis-Tsakonas et al., 1999).

Hematopoietic cells express both Notch transmembrane receptors and their ligands. The ligands such as Delta and Jagged are also expressed in stromal cells (Singh et al., 2000; Karanu et al., 2001; Artavanis-Tsakonas et al., 1995). Several studies have addressed the role of Notch signaling in hematopoiesis and have shown that one of the Notch receptors, Notch1 affects proliferation, differentiation and cell fate decisions of different types of hematopoietic cells at various stages of development (Pui et al., 1999; Stier et al., 2002; Ohisi et al., 2003). For example, Notch1 regulates the cell fate decision of CLP by promoting T-cell differentiation at the expense of B-cell differentiation (Stier et al., 2002). Furthermore during T-lineage maturation activated Notch1 promotes differentiation of cytotoxic T-cells, but not helper T-cells. In this case, constitutive expression of active form of Notch1 in hematopoietic progenitors results in production of few B-cells and T-helpers, but substantial number of cytotoxic T-cells (Radtke et al., 1999; Pear et al., 1996). In studies related to the adult hematopoietic system in the mouse, data from Duncan et al. (2005) have shown a key role for Notch signaling in the self-renewal of BM HSCs (inhibition of HSC differentiation).

To date, the function of Notch signaling in regulation of hematopoietic activity during development has been investigated in several knockout models. In Notch1-deficient embryos, hematopoietic neonatal repopulating activity in the E9.5 P-Sp and yolk sac was severely impaired. However, in the Notch2 -/- embryos no hematopoietic defects were observed, suggesting that Notch1, but not Notch2 plays important role in embryonic development of hematopoietic (stem) cells (Kumano et al., 2003).
In addition, it was recently shown that embryos, in which downstream transcriptional Notch co-activator RBPjκ was lacking, have impaired hematopoiesis. Moreover, coexpression of Notch1 and GATA-2 was observed at E10.5 in the endothelium lining the dorsal aorta and functional RBPjκ transcription binding sites were identified in the *GATA-2* promoter. The observation that Notch1 directly regulates GATA-2 expression and both molecules are expressed in the E10.5 dorsal aorta endothelium strongly suggests that Notch1 regulates AGM HSC activity via the transcriptional regulation of *GATA-2* (Robert-Moreno et al., 2005).

Hedgehog (Hh) signaling

Besides TGFβ, Wnt and Notch signaling, also members of the Hedgehog (Hg) family of patterning factors have been shown to regulate blood formation during mouse ontogeny (Murone et al., 1999; Byrd et al., 2002; Byrd and Grabel, 2004 review). The Hedgehogs are secreted proteins, which bind to the transmembrane receptor Patched and subsequently transmit a signal via Smoothened (Smo) proteins (Denef et al., 2000).

In the mouse and human there are at least 3 members of the Hedgehog family: Desert hedgehog (Dhh), Indian hedgehog (Ihh) and Sonic hedgehog (Shh). Two Hedgehog members, Ihh and Shh were shown to play a role in the induction of hematopoietic and vascular cell fates (McMahon et al., 2003; Dyer et al., 2001). Interestingly, Ihh was found to be expressed not only in the visceral endoderm of the yolk sac, but also implicated in the formation of yolk sac blood island and yolk sac angiogenesis (Byrd et al., 2002).

A clear role for Hedgehog signaling in blood vessel formation was shown in the Smo-deficient embryos. In these embryos no remodeling of the yolk sac vessels took place and the dorsal aorta in the anterior part of the embryo was absent (Zhang et al., 2001; Byrd et al., 2002; Vokes et al., 2004). Furthermore functional Hedgehog signaling studies from *Xenopus* embryos have shown that Hh signaling is required not only for proper development of dorsal aorta, but also for generation of definitive hematopoietic (stem) cells (Gering and Patient, 2005), which suggests an important role for Hh signaling in definitive hematopoiesis.

Another suggested, interesting aspect of Hedgehog signaling is its cross talk with other signaling pathways, including the Notch and VEGF pathways, which together with Hedgehog proteins take part in signaling cascades in embryonic angiogenesis (Byrd and Grabel, 2004 review). In addition, *in situ* hybridization data have shown that not only Ihh and Shh are coexpressed with BMPs (BMP-2, 4 and 6) in

the mouse embryo, but also BMP-4 is upregulated by Shh in human hematopoietic progenitors (Bitgood and McMahon, 1995; Bhardwaj et al., 2001). These data suggest that BMP-4 is one of the targets of the Hedgehog signaling and that both signaling pathways play an important role in hematopoiesis.

2. Hematopoietic transcription factors in murine blood development

A complex network of different factors regulates hematopoiesis throughout mouse development. These factors are represented not only by signaling molecules (already discussed), but also by transcription factors, which are capable of regulating the expression of target hematopoietic genes by binding to specific DNA sequences. Through activation of target genes, hematopoietic transcription factors can affect the proliferation, differentiation and/or self-renewal capacity of hematopoietic stem cells and progenitors. At different developmental and/or differentiation stages, the expression levels of hematopoietic transcription factors are particularly important, as the levels can affect the balance between these processes. A concise description of several hematopoietic transcription factors, selected on the basis of specific hematopoietic phenotypes in gene targeting studies, is presented here, with a particular emphasis on the roles played by these factors in hematopoietic stem/progenitor cell development.

2.1. GATA family

GATA transcription factors have been implicated in the development of several different hematopoietic lineages and hematopoietic stem cells. This family of factors includes 6 members, all of which contain conserved zinc finger motifs, which bind to the consensus DNA sequence (A/T) GATA (A/G) (Merika and Orkin, 1993; Yamamoto et al., 1990; Martin and Orkin, 1990; Orkin, 1992). Whereas the first three GATA factors (GATA-1, -2 and -3) are expressed mainly in hematopoietic system (Weiss and Orkin, 1995), GATA-4, -5 and -6 are expressed in non-hematopoietic organs such as intestines and lungs (Molentkin et al., 2000; Patient and McGhee, 2002). The GATA-1, -2 and -3 factors all of which play essential roles in development of hematopoietic cells, will be described in the following section.

2.1.1. GATA-1 regulates differentiation of hematopoietic cells

GATA-1 is also known as NF-E1, NF-1, Ery-1 and GF-1. With respect to the hematopoietic system, GATA-1 is expressed in multipotent progenitors (Shivdasani and Orkin, 1996), mature erythrocytes (Leonard et al., 1993), megakaryocytes, esosinophils (Iwasaki et al., 2005), mast cells (Martin et al., 1990) and dendritic cells (Gutierrez L., PhD thesis). GATA-1 was shown to affect development of different types of hematopoietic cells (Shivdasani et al., 1997; Yu et al., 2002; Kulessa et al., 1995). For

example, in mast cell precursors the expression level of GATA-1 is very low, but in mature mast cells GATA-1 levels are more abundant (Harigae et al., 1998). In addition, GATA-1 mutant mice with downregulated GATA-1 expression show a high number of mast cell precursors, but normal levels of (albeit morphologically aberrant) mast cells (Migliaccio et al., 2003). Overexpression of GATA-1 in this mutant restores the proper maturation of mast cells, indicating that GATA-1 directly regulates mast cell maturation.

In addition, GATA-1 was shown to play a critical role in the regulation of erythropoiesis. *In vitro* differentiation studies of ES cells lacking GATA-1 have demonstrated a block in the late stages of erythroid differentiation (at the proerythroblast stage), followed by apoptosis (Weiss et al., 1994). Chimeric mice generated with these GATA-1 deficient ES cells confirm the *in vitro* results, while other hematopoietic lineages were not affected (Pevny et al., 1991). However, as expected from erythroid arrest, GATA-1 knockout mice die at E10.5-E11.5 from severe anemia (Fujiwara et al., 1996). Similarly, mouse mutants with a knockdown of GATA-1 (GATA1.05) (Takahashi et al., 1997) and a GATA-1 low mutant (expressing about 20% of normal GATA-1 levels; McDevitt et al., 1997) die mostly at midgestation, with some of the GATA-1 low mice being born with severe anemia and surviving until adulthood (McDevitt et al., 1997; Shivdasani et al., 1997). Interestingly, overexpression of GATA-1 in murine erythroid progenitors affects their terminal maturation and leads also to anemia and embryonic death (Whyatt et al., 2000), which indicates a dose-dependent effect of GATA-1 in the development of erythroid cells.

GATA-1 plays an important role not only in differentiation, but also in the proliferation and survival of hematopoietic cells. Several target genes of GATA-1 are implicated in cell survival and cell cycle regulation (Rylski et al., 2003; Tanaka et al., 2000). For example, Bcl-2, an anti-apoptotic protein was shown to be directly regulated by GATA-1 (Weiss et al., 1997). Considering that overexpression of Bcl-2 in mice both at embryonic and adult stages leads to increased viability and activity of hematopoietic stem/progenitor cells, it is possible that GATA-1 is also involved, together with Bcl-2, in the survival of these cells (Domen et al., 2000; Orelio et al., 2004). Hence, GATA-1 may act not only during the late stages of differentiation of hematopoietic cells, but also at very early stages in multipotent hematopoietic cells.

2.1.2. Regulation of hematopoietic stem/ progenitor cell development by GATA-2

GATA-2 was identified on the basis of its homology to GATA-1 (Yamamoto et al., 1990). GATA-2 is highly expressed in hematopoietic multipotent progenitors, and its expression decreases with the maturation of these progenitors to more committed cells (Orlic et al., 1995; Persons et al., 1999).

In addition, high expression of GATA-2 in hematopoietic multipotent cells at embryonic and adult developmental stages has suggested a possible role of GATA-2 in the regulation of these immature hematopoietic cells. Analysis of GATA-2 expression

in the mouse embryo (through the use of GATA-2 GFP trangenic mice) has shown that GATA-2 is expressed in hematopoietic embryonic sites such as the P-Sp/AGM from E9.5 onwards (Minegishi et al., 1999; Kobayashi-Osaki et al., 2005). It is in this site where the first adult type of HSCs arise autonomously at E10.5 (Müller et al., 1994; Medvinsky and Dzierzak, 1996). These GFP positive cells coexpress HSC-specific markers such as c-kit, CD34 and CD45, suggesting that they possess HSC activity. GATA-2 expression was also observed in the FL and embryonic placenta (Minegishi et al., 1999; Ma et al., 1997), where HSC activity has also been detected (Ottersbach and Dzierzak, 2005; Gekas et al., 2005). Thus, GATA-2 appears to be an important regulator of HSC activity during embryogenesis. In addition, *GATA-2* levels have been found to affect an expansion of AGM HSCs. This will be discussed in more detail in **Chapter 4**.

GATA-2 was shown not only to play an important role in the regulation of HSC activity, but it also has an essential impact on hematopoietic differentiation. GATA-2-deficient ES cells failed to contribute to any definitive hematopoietic lineages in chimeric mice (Tsai et al., 1994; Tsai and Orkin, 1997). Examination of embryos deficient in GATA-2 has shown that they exhibit a hematopoietic phenotype, including severe anemia, which leads to embryonic lethality between E10.5-E11.5 (Tsai et al., 1994; Minegishi et al., 2003). Interestingly, the primitive erythrocytes remains morphologically normal, but a significant reduction in total number of red blood cells is observed in embryonic blood from the E9.5 embryo as well as in the YS (Tsai et al., 1994).

Moreover, data obtained from *in vitro* studies of GATA-2 deficient ES cells showed not only a large reduction in the number of definitive erythroid colonies, but also in mast cell colonies (Kulessa et al., 1995), suggesting that GATA-2 plays an important role in differentiation of both erythroid and mast cells. As GATA-2 expression declines during the differentiation of these different types of cells (Yamamoto et al., 1990; Harigae et al., 1998), simultaneous upregulation of GATA-1 in erythroid maturation is observed. In the case of erythroid differentiation, GATA-2 expression decreases as GATA-1 levels increase (Yamamoto et al., 1990). However, GATA-2 expression increases by 50-fold in erythroid cells, derived from GATA-1 deficient ES cells (Weiss et al., 1994). Despite this interplay between GATA-1 and GATA-2 in the regulation of hematopoietic differentiation, the absence of GATA-2 leads to a wider hematopoietic defect than GATA-1, since in contrast to GATA-1 lack of GATA-2 affects multipotent hematopoietic (stem) cells.

With regard to the adult hematopoietic system, GATA-2 is expressed in BM cells (Minegishi et al., 1999). Overexpression of GATA-2 in multipotent hematopoietic cells (Sca-1^{+}Lin^{-}) from BM resulted in decreased numbers of multipotent progenitors (CFU-S) *in vivo* without any signs of increased apoptosis. Furthermore, in transplantation assays these BM cells failed to provide multilineage reconstitution of adult recipients, which showed a reduced total number of Sca-1^{+}Lin^{-} cells compared to injected control BM cells (Persons et al., 1999; Heyworth et al., 1999). Therefore,

overexpression of GATA-2 in BM progenitors leads to an inhibition of both proliferation and differentiation of hematopoietic stem/progenitor cells. Similarly, a haploinsufficiency of GATA-2 in the adult hematopoietic stem/progenitors cells also results in their defective proliferation (Ling et al., 2004; Rodrigues et al., 2005). Thus, GATA-2 levels seem to be crucial during mouse development for preserving the pool of immature hematopoietic cells, preventing their exhaustion. Moreover, although both GATA-1 and GATA-2 regulate hematopoietic differentiation, the lack of GATA-2 affects hematopoietic stem/progenitor cells and all definitive hematopoietic lineages, which is not the case for GATA-1.

2.1.3. Role of GATA-3 in (definitive) hematopoiesis.

GATA-3, similar to GATA-2, was identified by its homology to GATA-1 (Yamamoto et al., 1990). GATA-3 was found to be expressed in T- and NK-cells and implicated in their development and function (Hattori et al., 1996; Hendriks et al., 1999; Samson et al., 2003; Pai et al., 2003).

GATA-3 plays an essential role in definitive but not in primitive hematopoiesis. GATA-3-deficient embryos suffer from internal haemorrhages, impaired FL (definitive) hematopoiesis and severe developmental defects in the nervous system and die between E11-E12 (Pandolfi et al., 1995). YS (primitive) hematopoiesis was normal in these embryos, as expected, as GATA-3 is not expressed in this tissue. In contrast to the YS, GATA-3 is expressed in the FL. Indeed, already from E9.5-E10 GATA-3 positive cells could be found in the septum transversum (precursor tissue of the FL), before colonization by hematopoietic (stem) cells (Manaia et al., 2000).

GATA-3 expressing cells were also found in the AGM region. Clusters of GATA-3 positive cells were found within mesenchyme underlying the dorsal aorta between E8-E11.5. At E10, expression of GATA-3 increased within these so-called subaortic patches (SAPs) (Manaia et al., 2000). Later on at E11-E11.5 GATA-3 expression was also observed in intraaortic clusters (hematopoietic intraaortic clusters; HIACs) on the ventral side of dorsal aorta (Bertrand et al., 2005). The presence of GATA-3 positive cells both in SAPs and HIACs at the developmental time when HSC activity can be detected in AGM region suggests that GATA-3 may be directly involved in the generation of definitive HSCs. Further studies by Bertrand et al., have shown that these GATA-3 positive cells found in SAPs and HIACs coexpress also other hematopoietic transcription factors such as GATA-2 and Lmo-2 and some cell-surface markers such as CD41 and CD31. Furthermore, the lack of expression of the hematopoietic marker CD45 in GATA-3 expressing cells initially suggested that the GATA-3 positive cells represent stromal cells (Manaia et al., 2000). However, *in vivo* transplantation data have shown that limited (when cells are injected into alymphoid recipient) HSC activity was found in the CD45 negative fraction of the AGM (at E10.5) (Bertrand et al., 2005). Thus, collectively all these data suggest that GATA-3 expressing cells observed in the AGM region possess some low level of HSC potential, and that

GATA-3 together with GATA-2 may play an important role in the generation of definitive HSCs in the AGM region.

Interestingly, recently published microarray expression profiles of murine adult BM populations have shown that GATA-3 was upregulated in LTR-HSC fraction as compared to the STR-HSC fraction (Zhong et al., 2005). Thus, it has been suggested that GATA-3 may not only be involved in the generation of embryonic HSCs, but also of adult HSCs.

2.2. SCL – a member of bHLH family

The family of transcription factors with a basic helix-loop-helix (bHLH) motif consists of members that play important roles in the regulation of different developmental processes such as hematopoiesis and neurogenesis (Massari and Murre, 2000; Lee et al., 1995). The bHLH family includes ubiquitously expressed members, as well as some factors, which are tissue-specific. Some of these latter ones, such as SCL (also known as Tal-1 or Tcl-5) was shown to play an essential role in primitive and definitive hematopoiesis (Begley and Green, 1999). Besides, SCL has been found to be associated with chromosomal translocations observed in human acute T-cell leukemias (Murre et al., 1989; Begley et al., 1989a).

SCL is expressed in different types of hematopoietic cells, including mature and multipotent cells (Begley et al., 1989b). SCL is also expressed in vascular endothelial cells as early as E8.5. A 3' enhancer element of the *SCL* gene was identified as being important for SCL expression in both cell types (endothelial and hematopoietic) in the embryo, and in the adult hematopoietic (progenitor) cells (Sanchez et al., 1999). With regard to multipotent hematopoietic cells, knock in of a single copy of *lacZ* reporter into the *SCL* locus in mice has shown that SCL is expressed within the midgestation embryo in HIACs as well as in the FL (E11.5) (Elefanty et al., 1999), where HSC activity is known to be found. In addition, other studies have demonstrated that expression of SCL in the FL increased by E14.5, which could be related to rising hematopoietic activity of this organ at this embryonic stage (Kallianpur et al., 1994). Therefore, SCL may play a role already at early stages of hematopoietic cell development. However, a function of SCL at later stages of definitive erythroid maturation was also described (Aplan et al., 1992). Thus, SCL could be a major hematopoietic regulator during both early and late events of hematopoietic differentiation.

A critical function of SCL in hematopoiesis was also demonstrated by knockout approach. SCL-deficient mouse embryos die around E9.5 due to very severe hematopoietic failure such as lack of major blood vessels and more importantly, the absence of YS hematopoiesis leading to a block in blood cell production (Robb et al., 1995; Shivdasani et al., 1995). In addition, these embryos are very pale and show growth retardation. However, the early stages of organogenesis appear not to be affected (Robb et al., 1995). Nevertheless the complete block in blood generation, resulting in a so-called bloodless phenotype, is confirmed by *in vitro* colony assays.

E9.5 YS cells or ES cells lacking SCL failed to produce any hematopoietic colonies. Furthermore, the SCL-deficient ES cells do not contribute to the hematopoietic system in chimeric embryos/adults, generated by injection of SCL $^{-/-}$ ES cells into wild type host blastocysts (Rob et al., 1995; Porcher et al., 1996).

Interestingly, SCL deletion results in the absence of transcripts of the hematopoietic transcription factors GATA-1 and PU.1 within YS and the embryo (Robb et al., 1995). It is possible that SCL may regulate the expression of GATA-1 and PU.1, the latter of which has been shown to be involved in myelo/lymphoid development (Scott et al., 1994). SCL was also shown to be critical for c-kit expression and c-kit functioning in the TF-1 hematopoietic cell line, suggesting that SCL may also act upstream of c-kit (Krosl et al., 1998). In this case SCL-dependent c-kit expression might affect the hematopoietic stem cell compartment, since c-kit is expressed on hematopoietic stem/progenitor cells. Thus, the presence of SCL is important for proper development of primitive and definitive hematopoietic cells. Although SCL plays an important role in the development of primitive and definitive hematopoietic cells at embryonic stages, adult SCL-deficient BM cells are still able to repopulate the hematopoietic system of adult recipients, suggesting that SCL activity is not required for the development of adult hematopoietic (stem) cells (Mikkola et al., 2003).

As compared to GATA-deficient mouse embryos, the hematopoietic defect in SCL-deficient embryos seems to be more severe and arises earlier in development. *In vitro* studies using SCL-inducible ES cells, where SCL expression was activated by tamoxifen, have demonstrated an essential function of SCL in the commitment of mesoderm to the hematopoietic lineage (Endoh et al., 2002). Hence, the activity of SCL may not only be restricted to hematopoietic cells during mouse embryogenesis but may affect mesodermal precursors at even earlier stages. Thus, SCL represents one of the most essential hematopoietic transcription factors, the activity of which is important for the proper development of murine hematopoietic (stem) cells particularly at embryonic stages.

2.3. A transcription factor essential in definitive hematopoiesis – the case of Runx1

Runx1 (also known as AML-1 or CBFA2 or PEBP2αB) was identified in chromosomal translocations in different types of leukemias. Runx1 is a member of CBFAα family, which contains two other members, CBFA1 (Runx2) and CBFA3 (Runx3) (Adya et al., 2000). All the members share a highly conserved runt domain, which binds DNA and interacts with the partner transcription factor, CBFβ (core-binding factor β) (Speck et al., 1995). In these heterocomplexes CBFβ stabilizes DNA binding ability of the Runx subunit and is involved in the regulation of its transcription (Tahirov et al., 2001).

The role of Runx1 in embryonic hematopoiesis was suggested by its spatio-temporal expression in embryonic tissues. The analysis of Runx1 lacZ knockin embryos has shown expression of Runx1 in the major arteries (vitteline and umbilical), FL and

AGM region at the time when HSC activity can be detected in these tissues. A more detailed analysis of Runx1 expression within the AGM revealed Runx1 positive cells not only in HIACS on the ventral part of the dorsal aorta, but also in the mesenchyme underlying the dorsal aorta (North et al., 1999), although not in the SAPs. Both of these regions were shown to contain cells with HSC phenotype and function (Bertrand et al., 2005). Interestingly, in embryos lacking Runx1 no HIACs have been observed, strongly suggesting that Runx1 together with CBFβ might be required for the generation of embryonic definitive HSCs. Furthermore, the analysis of HSC activity in the AGM region from Runx1-deficient embryos has confirmed a lack of functional HSCs in the AGM (Cai et al., 2000). In addition, transplantation studies with sorted, lacZ positive, AGM and FL cells from midgestation Runx1 lacZ knockin embryos have shown that HSCs providing long-term repopulation are Runx1 positive and coexpress other HSC markers such as c-kit and CD34 (North et al., 2002). Embryos carrying only one copy of *Runx1* were observed to have a decrease of HSC activity in the AGM (Wang et al., 1996; Cai et al., 2000), suggesting that both copies of *Runx1* are critical for optimal production of HSCs in the AGM (Robin et al., 2003).

Importantly, the critical role of Runx1 in definitive hematopoiesis was confirmed not only by expression studies, already described, but also by the phenotype of the Runx1 knockout embryos, showing massive haemorrhages and dying by E12.5 due to absence of definitive (FL) hematopoiesis (Okuda et al., 1996). However, primitive hematopoiesis was normal in these embryos, suggesting that Runx1 is an important regulator of definitive, but not primitive hematopoiesis. These data were further supported by results from studies on Runx1-deficient ES cells, which failed to contribute to definitive myelo/monocytic hematopoietic lineages *in vitro* and did not contribute to any definitive hematopoietic lineages *in vivo* (Okuda et al., 1996).

Runx1 was also shown to be implicated in adult hematopoiesis. Runx1 expression was detected in the majority of adult hematopoietic cells, including multipotent progenitors (CFU-S) and HSCs (North et al., 2004). Although Runx1 was detected in all definitive hematopoietic lineages (even at the single cell level) (North et al., 2004), others showed that Runx1 is not essential for the development of all of the definitive lineages in the adult (Ichikawa et al., 2004). Using a conditional Runx1 knockout, it has been shown that in the absence of Runx1 definitive hematopoietic lineages, including myeloid and erythroid are maintained. Furthermore, transplantation of Runx1-deficient adult BM cells revealed short-term (for at least 3 months) repopulation. However, transplanted $Runx1^{-/-}$ BM cells enriched in progenitors or HSCs failed to provide long-term multilineage reconstitution of the hematopoietic system of the recipients (Ichikawa et al., 2004). Thus, further studies on Runx1 conditional knockouts need to be performed to fully elucidate the role of Runx1 in the generation of adult HSCs.

Moreover, it would be interesting to further analyse the direct precursors to the Runx1 positive cells in the embryo and examine their relationship with the AGM HSCs by performing lineage tracing experiments.

3. Multiple origin(s) of murine hematopoietic stem cells – fate mapping

During embryonic and adult stages the hematopoietic system undergoes changes in anatomical localization. It also undergoes changes within its differentiation hierarchy, thus challenging our understanding of the lineage relationships of hematopoietic cells that appear during embryogenesis and those that are present in the adult. Since the conventional hematopoietic hierarchy does not appear to exist in the embryo until the first adult repopulating HSCs are generated at E10.5 in the AGM region, it is intriguing to investigate whether the hematopoietic stem/progenitor cells generated within the embryo contribute to the adult pool of multipotent hematopoietic cells or each hematopoietic pool is produced separately. Thus, the following section will describe cell lineage tracing approaches that may yield insight into the lineage relationships between embryonic HSCs or progenitors and the hematopoietic hierarchy found in the adult.

3.1. Are HSCs in adult BM coming from HSCs generated during embryogenesis?

Beginning in the 1970's Moore and Metcalf demonstrated the presence of hematopoietic progenitor cells both *in vitro* and *in vivo* in the YS, the first site in which differentiated erythroid cells are found. Importantly, they reported that HSC activity could be found in the YS beginning at E11. The YS was then postulated to be the first and primary hematopoietic site containing adult repopulating HSCs, which colonized the liver during fetal stages of development and subsequently the adult BM (Moore and Metcalf, 1970). These results were interpreted by the general hematological community as a demonstration that YS-derived cells are the only cells that contribute to adult (definitive) hematopoiesis. Additional experiments performed on the chick or murine YS (Moore and Owen, 1967b; Moore and Metcalf, 1970) further supported this early hypothesis about the extraembryonic origin of adult hematopoietic system (Moore and Owen, 1967a).

Interestingly, in parallel to studies on the embryonic origin of mammalian hematopoietic system, other vertebrate species such as amphibians and avian were also used as animal models to examine the origin of the hematopoietic system (LeDouarin, 1973; Dieterlen-Lievre, 1975; Kau et al., 1983; Turpen et al., 1976). Particularly, the *ex utero* development of these vertebrates served as the main advantage in fate mapping of the hematopoietic system during development. Orthotopic grafting, where the quail embryo was placed onto a chick blastodisc (YS analog) demonstrated that adult hematopoietic cells originated predominantly from the quail body. The chick yolk sac of this chimera gave rise only to transient hematopoietic cell populations in the embryo (Dieterlen-Lievre, 1975). More recent labelling of individual blastomeres in *Xenopus* embryo performed by microinjection of β-galactosidase RNA (into single blastomeres) resulted in tracing the origin of adult hematopoietic system to the intraembryonic part of the embryo. It was shown that the DLP (dorsal lateral plate; intraembryonic part of the

embryo; AGM equivalent) comes from distinct blastomere than the VBI (ventral blood islands; YS equivalent) and gives rise to adult type hematopoietic cells (Ciau-Uitz et al., 2000). Thus, already at this very early (32-cell) stage in *Xenopus* development, the mesodermally derived VBI and DLP regions are separated and will independently and autonomously contribute respectively to embryonic and adult hematopoietic compartments.

The hypothesis that the origin of the mammalian adult hematopoietic system was also from an intraembryonic site was first supported by experiments showing that CFU-S and B-lineage cells could be found in a region containing the dorsal aorta (Medvinsky et al., 1993; Godin et al., 1993; Marcos et al., 1994). In early gestation this mesodermally derived region is called the para-aortic splanchnopleura (P-Sp) and slightly later E9-E13 is called the aorta-gonad-mesonephros region (AGM). Multipotential hematopoietic progenitors were found to be autonomously generated in the P-Sp (but not in the YS) before establishment of the circulation at E8.5 (Cumano et al., 1996; Cumano et al., 2001). Later at E10.5 (34-40sp), the fist adult repopulating HSCs are found in the AGM region and are autonomously generated within it (34-40sp) (Medvinsky and Dzierzak, 1996; Müller et al., 1994). Careful analysis of HSC activity in murine AGM, YS, FL through different embryonic stages, beginning from E8 till E12, have shown an increase in HSC activity in the AGM with peak at E11/E12, when such adult repopulating HSCs are just beginning to appear in the YS and FL (Medvinsky and Dzierzak, 1996). To exclude the possibility of circulation of HSCs between different hematopoietic tissues, this set of experiments was performed using the explant cultures of whole hematopoietic organs. Such an explant culture system allows not only the examination of individual embryonic tissues, but allows analysis of the expansion of HSCs during the culture. It was shown that culture of E11 AGM explants increases HSCs by the factor 13 (Kumaravelu et al., 2002). Interestingly, further subdissection of E11 AGM revealed that HSC activity is detected in the dorsal aorta and surrounding mesenchyme, but not in the urogenital ridges at this stage (de Bruijn et al., 2000b). Importantly, among different hematopoietic tissues tested, the AGM represents the earliest hematopoietic site within the mouse conceptus where HSCs are autonomously generated. However, according to quantitative data published by Kumaravelu et al., while the AGM contains more HSCs than YS, there is a great possibility that the YS may also be an independent site of HSC production.

Another recently discovered potent hematopoietic tissue, where HSCs may be *de novo* generated, is the midgestation placenta. HSC activity in the placenta starts to be detected, similarly to AGM, at E10.5 and reaching the peak at E12 with estimated number of twelve HSCs (Ottersbach and Dzierzak, 2005). Interestingly, quantitative HSC analysis of different embryonic hematopoietic organs has shown that embryonic placenta contains significantly more HSCs than circulating blood and more importantly, HSCs appear earlier in the placenta than in the circulation (Ottersbach and Dzierzak, 2005; Gekas et al., 2005). Thus, placental HSCs do not simply represent multipotent cells found in the circulation, but interestingly these HSCs may be either produced in

the placenta or migrate as precursors to this potent hematopoietic niche (Gekas et al., 2005). These possibilities need further investigation.

In addition, although an expansion of HSCs in the placenta to an estimated fifty LTR-HSCs at E12.5 is one of the highest (Gekas et al., 2005), the explant culture of whole placentas failed to maintain or expand the HSCs during the culture period (Ottersbach and Dzierzak, 2005). *In vivo* examination of the HSC activity in the placenta at much earlier stages, at E9 or E10 would be a good approach to assess independent HSC generation. Such (transplantation) experiments were conducted but only with a limiting number of adult recipients and with no flow cytometric sorting of HSCs for enrichment purposes. Unfortunately, injection of 1 to 4 ee (1 to 4 tissues/recipient) did not result in detection of repopulating HSC activity (Ottersbach and Dzierzak, 2005). Nevertheless, it is still promising that the embryonic placenta may produce HSCs at the same time point or earlier than AGM. If so, the placenta will then replace the AGM as the first site where adult repopulating HSCs are autonomously generated.

In summary, during mouse embryogenesis the only well documented region of adult type of HSC production to date is the AGM. However, such multipotent hematopoietic cells may also be generated or expanded in the extraembryonic hematopoietic tissues such as the YS and the placenta. In this scenario, it is also probable that HSCs may migrate between all these three different organs, followed by colonization of the FL and finally colonization of the BM in the adult (**Fig.6**). Thus, to precisely define different sites of origin and routes of HSC migration during mouse development, lineage tracing approaches must be taken.

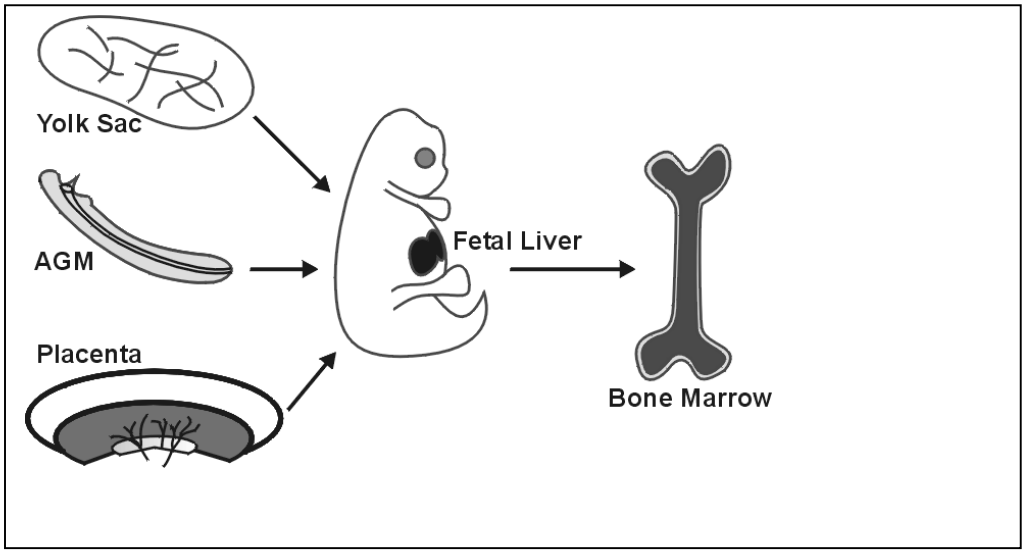

Figure 6. Proposed model of migration of HSCs during mouse development.
HSCs within the mouse conceptus can be not only generated or expanded in the intraembryonic part, the AGM region but also may be autonomously produced in the extraembryonic hematopoietic tissues such as the yolk sac and the placenta. Thus, in this scenario the HSCs from these 3 different hematopoietic organs would contribute to the HSC pool of the fetal liver, which remains hematopoietically active till the birth, when the BM becomes subsequently the central site of hematopoiesis in the adult (adapted from Ottersbach and Dzierzak, 2005).

3.2. Do immature HSCs exist? What are the direct precursor cells to HSCs?

The tracing of adult type of HSCs within the mouse conceptus remains not only an interesting challenge to define the origins and migration of HSCs during ontogeny, but also lineage tracing will help to find the direct precursor cells to HSCs. The fact that development of HSCs occurs in very close contact with surrounding vascular endothelium and many molecular markers are shared between hematopoietic and endothelial cells (Wood et al., 1997) brought a large interest with regard to HSC origin to the hypothesized bipotential mesodermal precursor named the hemangioblast. The hemangioblast by definition is able to give rise to hematopoietic and endothelial lineages (Sabin, 1917; Murray, 1932). While hemangioblasts have been observed *in vitro*, there is a little evidence of their existence *in vivo*. Using *in vitro* model of ES cell differentiation, cells equivalent to hemangioblasts were identified as blast-colony forming cells (BL-CFC), which appeared early (before hematopoietic cells) and transiently during ES cell differentiation culture and possessed both hematopoietic and endothelial potentials (Choi et al., 1993).

Based on mouse knockouts or ES cells lacking genes implicated in both hematopoietic and endothelial development, several factors (including transcription factors and signaling molecules) were shown to play an important role in hemangioblast formation. For example, deletion of SCL in mice leads to an absence of hematopoietic and endothelial cells in the YS (Robb et al., 1995; Shivdasani et al., 1995). Moreover, in differentiation cultures of SCL-deficient ES cells no BL-CFCs were found, suggesting a critical role of SCL in hemangioblast development (Robb et al., 1995). Similar to the SCL-deficient ES model, ES cells lacking the tyrosine kinase receptor Flk-1 failed to contribute to vascular endothelium and definitive hematopoietic cells (Shalaby et al., 1995). These data were further confirmed by gene targeting experiments in mice (Shalaby et al., 1995; Shalaby et al., 1997). Interestingly, the Runx1 hematopoietic transcription factor was also found to be required for production of normal numbers of BL-CFCs under *in vitro* conditions (Lacaud et al., 2002). Collectively, an identification of hemangioblasts in several ES cell differentiation systems provided supportive data that were extremely useful in further studies focused on detecting the hemangioblast *in vivo*.

Using a mesoderm-specific Brachury GFP reporter mouse, the first hemangioblasts were found in cell populations coexpressing GFP and Flk-1 in the embryos at the early stages of the gastrulation (mid-primitive streak) (Huber et al., 2004). Although the identification of such early appearing hemangioblasts (before formation of blood islands) during mouse embryogenesis casts doubt on the hypothetical hemangioblast, it is still challenging to further search for more specific types of hemangioblasts *in vivo*, which will give rise either to primitive or definitive hematopoietic lineages. Furthermore, since a large body of experimental data support the intraembryonic origin of mammalian adult hematopoietic system, it would be of great interest to identify a new type of hemangioblast within the intraembryonic region of the embryo (Dzierzak, 2005).

Intriguingly, fate mapping experiments performed on chick embryos shed light on this interesting aspect of HSC origin. Labelled endothelial cells in the precirculation aorta were found to give rise to hematopoietic cells that appeared shortly thereafter in clusters emerging from the dorsal aorta (Jaffredo et al., 1998). These results suggest the existence of a specific type of endothelium with hematopoietic potential, the so-called hemogenic endothelium (Jordan, 1916). Such hematopoietic clusters were also detected in the mouse conceptus in different hematopoietic regions, including major vessels such as the dorsal aorta and vitelline and umbilical arteries (de Bruijn et al., 2002). More importantly, these clusters of hematopoietic cells were found at the time when HSC activity is detected. They express markers characteristic for both hematopoietic and endothelial cells (de Bruijn et al., 2002; North et al., 1999; North et al., 2002), indicating that these adult type of HSCs in the embryo emerge from vascular endothelium lining the dorsal aorta. In contrast, recently described expression analysis of hematopoietic and endothelial markers in the SAPs and HIACs have shown the differential expression of some markers (GATA-3, CD41 and Flk-1) suggesting that HSC precursors exist in the SAPs and not in the endothelium (Bertrand et al., 2005). However, transplantation of sorted cells with endothelial characteristics and localization have shown that functional HSCs are found within the endothelial subpopulation of E11 AGM (North et al., 2002; de Bruijn et al., 2002). Thus, the discrepancies between hematopoietic potential of vascular endothelium and lack of direct evidence for existence of adult type of hemangioblast in the embryo requires more advanced and clonal methods that will enable researchers to clarify the relationship between these different types of HSC precursors involved in HSC development.

3.3. How to trace the hematopoietic (stem) cells during development?

Identification of various HSC precursors in the embryo without a defined relationship to adult type of HSCs triggered a large interest in the development of techniques allowing for marking of the cells at molecular level. These so-called fate mapping methods include usage of different labelling molecules. In the classical tracing studies distinct sort of markers are applied, such as vital dyes (Keller, 1975; Keller,

1976; Jaffredo et al., 1998), different molecular (retroviral or *in vitro* synthesized reporter RNA) (Cepko et al., 1993; Lawson et al., 1991; Zernicka-Goetz et al., 1997) or immunochemical markers (Pedersen et al., 1986; Lawson et al., 1986; Lawson and Pedersen, 1992). One of the latter ones such as HRP (horseradish peroxidase) appeared to be a very useful lineage tracer in generation of fate maps of all three germ layers. When HRP is injected into single epiblast cell of the prestreak and early streak mouse embryos, the migration of these cells can be followed in the gastrulating embryo after one day of *in vitro* culture (Lawson et al., 1991). Indeed, in these studies Lawson and co-workers found that the first emerging mesodermal cells migrated only to the extraembryonic yolk sac and not to the embryo body.

With regard to the origin of HSC, by using one of the vital dyes, DiI (LDL-DiI) it has been demonstrated that vascular endothelial cells surrounding the dorsal aorta of the chick embryo most likely represent the direct precursor cells to HSCs. In order to label the endothelial cells in the chick embryos, before emergence of intraaortic hematopoietic clusters, an injection of the acetylated LDL-DiI (acetylated low density lipoprotein binds preferentially to vascular endothelial cells) into the heart was performed. After approximately one day of *in vitro* embryo culture, histological analysis of the embryos sections have identified the DiI-labelled hematopoietic cells within the intraaortic clusters of the dorsal aorta (Jaffredo et al., 1998). Such DiI positive cells, expressing the hematopoietic marker CD45, were also detected in the mesenchyme underlying the dorsal aorta, the para-aortic foci. The previously mentioned SAPs may be equivalent to these para-aortic foci in the chick embryo. Because of the time limitations for DiI usage, additional retroviral labelling of the dorsal aorta endothelium before appearance of the hematopoietic clusters was performed in chick embryos. After two days of *in vitro* culture, histological examination of embryo sections revealed that similar to intraaortic clusters, the para-aortic foci also contained retrovirally marked cells. Interestingly, retroviral labelling at the time of disappearance of the intraaortic clusters, demonstrated an absence of marked cells within the para-aortic foci (Jaffredo et al., 2000). Thus, hematopoietic cells within both the intraaortic hematopoietic clusters and para-aortic foci derive from the aortic endothelium and are present only temporally, which seems to be the case also for the murine HIACs and SAPs (Bertrand et al., 2005).

Nevertheless, these above described types of classical tracing experiments of hematopoietic cells have some major limitations. The immunochemical markers as well as fluorescent dyes can only be applied exogenously and for a very short period of time, since for instance with every cell division dilution of fluorescent signal occurs. Hence, an ideal tracing of HSC fate through ontogeny would use a marker which can be detected for a prolonged time, be easily visualized *in vivo*, be applied to specific cells at a specific time and not disrupt the normal physiology or development of the embryo. Such a genetic labelling system has been already established with great success. Introduction of the Cre/loxP system avoids all technically difficult grafting or injection marking procedures. While it has great advantages, it also has its technical limitations.

The main advantage of the Cre/loxP fate mapping system is its simplicity, since only two components are required, Cre recombinase and lox recombination elements flanking a reporter gene (**Fig.7**). Originally the Cre recombinase was isolated from bacteriophage P1 (Abremski et al., 1983; Abremski et al., 1984). This 38-kD protein belongs to λ integrase family of site-specific recombinases, which perform recombination by binding to two specific 34-bp DNA sequences, named loxP sites (Sternberg and Hamilton, 1981; Hoess et al., 1986; Dymecki et al., 2000). The recombination mechanism is well conserved between different site-specific recombinases and includes a cleavage, exchange and ligation of DNA strands

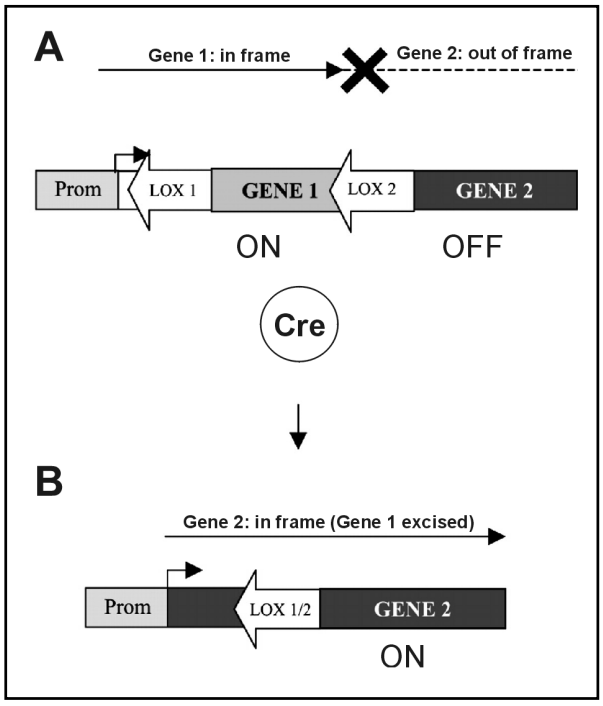

Figure 7. Genetic fate mapping by the Cre/loxP system. In the upper part (**A**) there is shown a schematic representation of reporter line construct where the reporter gene in the single reporters corresponds to GENE 2, in dual reporters- to GENE 1 and GENE 2. In the absence of Cre recombinase the GENE 2, encoding the recombination marker, is inactive, while the first reporter GENE 1 (dual reporters) is expressed in the cells where recombination did not occur. Cre-mediated recombination (**B**) between loxP sites (LOX 1 and LOX 2) leads to deletion of loxP-flanked GENE 1 and restoration of the open reading frame of the GENE 2, followed by the expression of the recombination marker.

(Sadowski et al., 1995; Lee and Sadowski, 2001). The final products of Cre-mediated recombination depend on orientation of the loxP sites. For example, if two loxP sites are positioned in the same direction (direct repeats), a DNA fragment flanked by them will be removed as a circular molecule. In contrast, recombination between two inverted loxP sites will lead to an inversion of the flanked DNA fragment (Sauer and Henderson, 1988; Sauer and Henderson, 1990).

Introduction of the Cre/loxP recombination technology into animal models, such as a mouse with specific orientation of loxP sites flanking the gene of interest, has allowed for functional *in vivo* studies of target genes (Rüffer and Sauer, 2002). Moreover, with regard to the tracing of particular type of cells during ontogeny, it was essential to generate different lineage-specific Cre transgenic lines and transgenic lines with reporter genes which expression was prevented by insertion of a STOP cassette

flanked by loxP sites. Thus, the reporter would be expressed only as a result of recombination event in cells expressing the Cre recombinase and their progeny. Cell tracing is then based upon the inheritability of the recombination marker in all the progeny of the originally recombined cell (Zinyk et al., 1998; Branda and Dymecki, 2004). To date many mouse reporter lines have been generated (**Table 2**), with various recombination markers including human placental alkaline phosphatase (PLAP), variants of GFP and β-galactosidase (Kisseberth et al., 1999). Also, double reporter lines such as Z/AP (Lobe et al., 1999) or Z/EG (Novak et al., 2000) have been generated. These lines were generated so as to facilitate the labelling of both unrecombined, as well as recombined cells. Besides improvements in a production of very sensitive reporter lines without any leaking activity in the absence of Cre recombinase, also mouse lines where Cre activity can be controlled in a temporal manner were produced. Such an inducible Cre/loxP system has become an attractive approach in marking specific cell types only within a narrow window of developmental time.

Table 2. Recombination reporter lines used in fate mapping of hematopoietic cells.

Type of reporter	Promoter/ regulatory sequences	Trangenic (T) or knock in (K)	Type of reporter gene	Reference
Rosa26 lox lacZ (R26R)	Rosa26	K	ß-gal	Soriano et al., 1999
Rosa26 lox EGFP	proviral Rosa26	K	EGFP	Mao et al., 2001
Rosa26 lox EYFP	Rosa26	K	EYFP	Srinivas et al., 2001
PGK lox EGFP (sEGFP)	PGK	T	EGFP	Gilchrist et al., 2003
CAG-CAT-lacZ	CAG	T	CAT/ß-gal	Araki et al., 1995
Z/EG	CAG	T	ß-gal/EGFP	Novak et al., 2000

PGK- phosphoglycerate kinase; CAG- chick ß-actin promoter connected with cytomegalus enhancer sequences; CAT- chloramphenicol acetyltransferase; PLAP- human placental alkaline phosphatase; EYFP- enhanced yellow fluorescent protein; Z- lacZ; ß-gal- ß-galactosidase; EGFP- enhanced green fluorescent protein (adapted from Branda and Dymecki, 2004).

3.3.1. Current status of Cre/loxP cell tracing experiments

To date, Cre/loxP technology has been applied in the tracing of different types of hematopoietic cells both *in vitro* and *in vivo*. *In vitro* studies using a B-lymphoid specific-Cre/loxP system have demonstrated that B cells can be reprogrammed into

functional macrophages (Xie et al., 2004). Such genetic labelling of the B-lymphoid cells enabled the study of lymphoid to myeloid conversion induced by overexpression of C/EBP and PU.1 transcription factors. Normally, all B cells ($CD19^+$) in double transgenic CD19 Cre: Rosa26 lox EYFP adult mice are YFP positive, while all Mac-1 expressing myelomonocytic cells are EYFP negative. However, $CD19^-$ $Mac\text{-}1^+EYFP^+$ cells were found upon *in vitro* culture of CD19-expressing spleen or BM cells retrovirally infected with C/EBP and PU.1. This B to myeloid cell conversion was furthermore confirmed by *in vivo* data. Transplantation of C/EBPα-infected B cells from adult BM (CD19 Cre: Rosa26 lox EYFP) demonstrated that at 6 days post transplantation, 51% of BM and 32% of spleen cells (expressing C/EBPα) have the marked macrophage phenotype ($CD19^-$ $Mac\text{-}1^+EYFP^+$).

Concerning still unresolved questions regarding HSC development and *de novo* generation or colonization of the adult blood system by embryonic hematopoietic multipotent cells, Cre/loxP system was also applied in fate mapping of multipotent hematopoietic cells *in vivo*. Thus far, several transgenic lines expressing either direct or inducible variants of Cre recombinase in hematopoietic system have been produced. One of the hematopoietic-specific Cre lines is the Mx1 Cre, which was used to induce knockouts of target genes in adult HSCs (Mikkola et al., 2003; Zhang et al., 2003; Ichikawa et al., 2004). Mx1 Cre was shown to efficiently delete genes in the entire hematopoietic system. A drawback of this Cre is that it is driven by the interferon α/β-inducible promoter of *Mx1* gene (Kuhn et al., 1995). It is thought that interferon administration disrupts the homeostasis of the hematopoietic system, perhaps inducing unwanted variables during hematopoietic studies. In addition, in healthy mice the Mx1 promoter is usually not active. However, due to the health status of experimental mice, endogenous interferon levels may rise thus promoting leaky Cre expression. Additionally, the recombination activity of Mx1 Cre was not only observed in hematopoietic tissues, but also in other non-hematopoietic organs (Akiyama et al., 2002; Kemp et al., 2004).

To target Cre expression specifically to fetal hematopoietic multipotent cells regulatory elements of *CD41* gene has been used (Emambokus et al., 2003). FDG-flow cytometry analysis of E14.5FL from compound double transgenic embryos (CD41 Cre: Rosa 26 lox lacZ), has shown that 44% of cells recombined (lacZ positive) and some of them express markers used for HSC enrichment such as c-kit, CD34 and CD41. In addition, histological examination of hematopoietic tissues showed lacZ expressing cells at E9.5 in the P-Sp region and that their number increased at E10.5 in the AGM. However, further immunostaining data showed only a partial overlap between CD41 and lacZ expressing cells. Nevertheless, recombined cells expressing lacZ were found in the fetal hematopoietic tissues at the time when HSC activity is detected, suggesting that efficient marking of fetal hematopoietic multipotent cells took place. Conversely, these fetal progenitor cells marked at embryonic stages failed to give any significant contribution to adult hematopoiesis. Unfortunately, no hematopoietic functional *in vivo* assays testing hematopoietic potential of these fetal cells were performed.

Effective targeting of hematopoietic multipotent progenitors (CFU-S) *in vivo* was demonstrated in studies, where a retroviral system expressing Cre under 3'SCL enhancer has been used (Murphy et al., 2003). Recently, this enhancer was also used in a Cre transgenic line to trace HSCs during mouse development (Göthert et al., 2005). In order to induce Cre-mediated recombination in multipotent hematopoietic cells at the time of HSC emergence, Tamoxifen-inducible Cre was placed under control of 3'SCL enhancer and the exon 4 of SCL promoter. To induce recombination in embryonic HSCs or hematopoietic progenitors, two consecutive i.p. (intraperitoneal) injections of Tamoxifen at E10.5 and E11.5 were performed in pregnant dams. Flow cytometric analysis of E14.5 FL cells from double transgenic (SCL-CreERT: Rosa26 lox EYFP) offspring showed that 3.9% of cells were recombined (EYFP positive). By applying additional HSC markers, this EYFP positive FL subpopulation was found to be highly enriched in HSCs, indicating that Cre-mediated recombination occurred in multipotent hematopoietic cells (marked at E10.5/E11.5). In addition, transplantation of these E14.5FL cells into adult irradiated recipients revealed that about 10% of donor-derived BM cells were $EYFP^+$. The same percentage of donor-derived, EYFP positive BM cells was obtained, when *in utero* Tamoxifen-treated (at E10/E11) double transgenic embryos were allowed to develop untill adulthood. Thus, for the first time it has been demonstrated that embryonic HSCs contribute directly to adult HSC pool, although with a low frequency. This minor contribution to the adult compartment can be explained in three ways: 1) unsuccessful marking at E10/E11 of FL cells; 2) *de novo* HSC production before/or after marking took place in the embryo or 3) independent generation of HSCs in the adult. Considering all these possibilities and the fact that efficiency of Cre-mediated recombination never reached so far 100%, the first explanation seems to be the most probable. However, further studies and optimizations should be performed.

With respect to the proposed endothelial origin of adult type of HSCs, there has been generated a transgenic line expressing Cre recombinase under control of the promoter of the *Tie-1* gene, which encodes the endothelial cell specific tyrosine kinase receptor (Korhonen et al., 1994; Dumont et al., 1995). Tie-1 was reported to be also expressed in HSCs (Iwama et al., 1993; Yano et al., 1997). Histological analysis of embryonic hematopoietic tissues from double transgenic embryos (*Tie-1* Cre: Rosa26 lox lacZ) has shown expression of the lacZ recombination marker not only in the vasculature of the YS (E8/E9), but also in the dorsal aorta endothelium at E10 (Gustafsson et al., 2001). Interestingly, lacZ expressing cells were also detected in the vascular endothelium of the labyrinth vessels of the embryonic placenta. Previously, GFP positive cells possessing HSC activity were found in the vascular labyrinth of the placenta in the Ly-6A GFP transgenic line (Ottersbach and Dzierzak, 2005). Recombined lacZ positive cells were also detected in adult hematopoietic organs such as BM, thymus and spleen isolated from the compound mice. Thus, *Tie-1* Cre-mediated recombination, followed by lacZ expression, occurred both in embryonic and adult hematopoietic organs at the time when HSC activity has also been found.

Unfortunately, coexpression of HSC-markers was not analyzed and no transplantation studies of these recombined cells were performed. Hence, based on the murine studies it still remains unclear if genetically marked endothelial cells have hemogenic potential. Only (ligand)-inducible Cre/loxP genetic marking of HSCs may reveal more details with regard to HSC origin and development during mouse ontogeny.

Scope of the thesis

Chapter 1 of this thesis contains an overview of the murine hematopoietic system and its generation and expansion during embryonic, fetal and adult stages. Particularly, with regard to development of HSC and regulation of its activity during mouse ontogeny in the various anatomic hematopoietic sites, transcription factors and signaling networks are described. Up to date information concerning the origin(s) of adult type of HSC and the lineage relationship between HSC precursors and HSCs is also included here. *De novo* generation of HSCs in embryonic and adult hematopoietic sites or contribution of embryonic HSCs to adult hematopoiesis is an ongoing question. Thus, one of the aims of the work described in this thesis was to trace the hematopoietic stem cells from the embryo through to the adult using Cre/loxP recombination marking system.

In **Chapter 2** the results obtained from such hematopoietic stem/progenitor cell fate mapping experiments performed both *in vitro* and *in vivo* are presented. To target the multipotent hematopoietic cells we have used the Ly-6A CreERT transgenic line, which expresses Tamoxifen/4OHT-inducible Cre recombinase under control of regulatory elements of the *Ly-6A* gene. Previously, these regulatory elements of the *Ly-6A* gene have been used in generation of the Ly-6A GFP mouse line, where *GFP* transgene (expression) appeared to be a very reliable marker for all functional HSCs both in embryo and in the adult. In an attempt to label these HSCs at different developmental stages, two different lox reporter lines (Rosa26 lox lacZ and Rosa26 lox EGFP) were applied. Thorough examination of recombination (Tam/4OHT-dose and time-dependent) in both fetal and adult hematopoietic sites was performed.

Chapter 3 describes studies focused on neonatal repopulating cells, using the W^{41}/W^{41} neonate as a recipient model. It has been suggested that such neonatal repopulating cells may be direct HSC precursors. We were particularly interested in determining whether E9/or E10 embryonic hematopoietic tissues (P-Sp or YS) isolated freshly or from *in vitro* cultured embryos are able to repopulate the hematopoietic system of unconditioned W^{41}/W^{41} newborns. In addition, to study the neonatal repopulating activity by transplantation assays W^{41}/W^{41} mutants appear to be an interesting recipient, since the c-kit mutation in these W mutants affects not only phenotype, but also, more importantly, HSC potential.

In turn, in **Chapter 4** the role of GATA-2 in generation and expansion of definitive HSCs during mouse development is investigated. In addition, since gene

dosage of certain transcription factors was shown to affect activity of the adult type of HSCs in the embryo, it was motivating and interesting to examine also the effect of *GATA-2* levels in HSC development *in vivo* and *in vitro* both at embryonic and adult stages. These data are presented in **Chapter 4**.

Chapter 5 contains a general discussion, where all obtained results regarding various aspects of HSC development are included and considered. Furthermore, future prospects related to studying the molecular events of HSC emergence and HSC development in mouse ontogeny are also given in this chapter.

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CHAPTER 2

Fate mapping of hematopoietic stem cells (HSCs) during mouse development

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INTRODUCTION

The origin of the mammalian adult hematopoietic system, and in particular the precise embryonic site from which it is derived, remains unclear. Initially it was thought that the adult blood system or its precursors are generated in the yolk sac, and then colonize the fetal liver and subsequently the adult bone marrow (Moore and Metcalf, 1970). However, according to results obtained from grafting and lineage tracing experiments performed in non-mammalian vertebrates (Dieterlen-Lievre, 1975; Turpen et al., 1981; Ciau-Uitz et al., 2000), embryonic and adult hematopoietic systems originate independently within the embryo. Adult type of blood precursors and long-lived hematopoietic stem cells (HSCs) are generated in an intraembryonic region containing the dorsal aorta, while the short-lived embryonic blood cells arise in the yolk sac. Due to the inability to manipulate *in utero* developing mammalian embryos, only indirect studies have been performed to examine the origin of the adult mouse hematopoietic system. In the mouse embryo, the first transplantable adult-type HSCs are autonomously generated at E10.5 in the intra-embryonic aorta-gonad-mesonephros (AGM) region. From E11 onwards such HSCs can be found in the yolk sac and fetal liver (Müller et al., 1994; Medvinsky and Dzierzak, 1996). Thus, the AGM is the first and most potent embryonic site of adult HSC formation in a mammal, strongly suggesting that this intraembryonic site contributes to the adult hematopoietic system.

Experiments quantitating the number of HSCs throughout the midgestation mouse embryo suggest that AGM-derived HSCs migrate to both the yolk sac and fetal liver (Kumaravelu et al., 2002). These data and data obtained from a haploinsufficient *Runx1* and *GATA-2* midgestation embryos also strongly suggest that the yolk sac can autonomously produce HSCs and that these yolk sac-derived HSCs contribute to the colonization of the fetal liver and, thereafter, the adult bone marrow. Most recently, another site of potent HSC activity has been found in the vascular labyrinth region of the midgestation placenta (Ottersbach et al., 2005; Gekas et al., 2005). Thus, multiple sites within the developing mouse embryo may be a source of the adult hematopoietic system. In addition, rather than migration of embryo-derived HSCs to adult hematopoietic sites, HSCs may be *de novo* generated within the fetal and/or adult hematopoietic sites.

To investigate whether the adult hematopoietic system is derived from HSCs generated within the midgestation mouse embryo, Cre/loxP recombination systems offer the best currently available method for cell lineage tracing. The inducible Cre/loxP system has been already tested with great success in *in vitro* culture systems and in the generation of conditional mouse knockouts (Vallier et al., 2001; Metzger et al., 1995; Tannour-Louet et al., 2002). With the use of tissue-specific promoters, the expression of inducible Cre (CreERT) can be controlled both temporally and spatially, thus allowing very specific recombination events (Danielian et al., 1998; Vasioukhin et al., 1999; Watt et al., 2001; Leone et al., 2003). For CreERT, the nuclear localization of Cre recombinase (and thus its activity) is reliant upon the mutated ligand-binding domain of

estrogen receptor (ERT) to which it is fused. The mutation G525R in the murine ligand-binding domain (LBD) results in a decreased (about 1000-fold) binding affinity of a natural ligand, 17β-estradiol to LBD, but increased binding of synthetic ligands such as Tamoxifen or 4-hydroxytamoxifen (4OHT) (Danielian et al., 1993; Danielian et al., 1998). Thus, Cre-mediated recombination should occur only in the presence of Tamoxifen. Based on this Tamoxifen-inducible CreERT/loxP system, it should be possible to recombine a loxP-flanked marker gene in HSCs *in vivo* only during the short window of developmental time, when the first HSCs appear in the AGM region (at E10.5). To date the CD41 (Emambokus et al., 2003) and SCL (Göthert et al., 2004) promoters have been used to direct the expression of Cre recombinase (or Tamoxifen inducible CreERT) to hematopoietic cells: CD41 is expressed in hematopoietic progenitors and SCL is expressed in endothelial and hematopoietic cells. These Cre transgenic mice were crossed with Rosa26 lox lacZ (Soriano et al., 1999) or Rosa26 lox EYFP (Mao et al., 2001) mice. In the case of CD41 Cre, lacZ expressing cells were found in the fetal liver but only very few in the adult bone marrow, suggesting that there is no or little migration of hematopoietic cells to the bone marrow or that CD41 is expressed only in short-lived hematopoietic cells. In the case of SCL CreERT and E10/11 Tamoxifen *in utero* treated offspring, YFP expressing cells were found both in the fetal liver and the adult bone marrow, suggesting migration and colonization of the adult bone marrow by E10/11 marked hematopoietic cells. However, due to the wide expression pattern of SCL it is uncertain whether the fetal/adult marked cells are the outcome of migration of HSCs from the AGM.

Previously, the *Ly-6A* (Sca-1) transcriptional regulatory elements have been demonstrated to direct the expression of a GFP marker gene to all HSCs within the AGM, fetal liver, placenta and adult bone marrow (Ma et al., 2002; de Bruijn et al., 2002). Indeed, it is one of the most specific markers of the first HSCs emerging within the ventral aspect of the midgestation dorsal aorta (de Bruijn et al., 2002). Thus, for the specific expression of CreERT within the first emerging HSCs, we generated and established a transgenic mouse line, Ly-6A CreERT with a high expression of Tamoxifen-inducible Cre recombinase in hematopoietic tissues.

To perform fate mapping experiments and *in vivo* mark HSCs we established matings of Ly-6A CreERT males with Rosa26 lox lacZ and Rosa26 lox EGFP reporter line females. Upon Cre-mediated recombination the offspring should express ß-galactosidase and EGFP, respectively (Soriano, 1999; Mao et al., 2001). Pregnant females were treated with Tamoxifen at E9, E10 or E11 to induce recombination *in utero* within the AGM HSCs. For induction of the Cre-mediated recombination in fetal liver HSCs, females were treated with Tamoxifen at E13/14. We report here our results showing a small number of reporter expressing cells both in embryonic and adult hematopoietic tissues of such offspring. Recombination appeared to occur only at a very low frequency. Thus, we are unable to clearly determine whether AGM HSCs migrate and colonize adult hematopoietic tissues.

RESULTS

GENERATION OF Ly-6A CreERT TRANSGENIC MICE AND TRANSGENE EXPRESSION IN THE EMBRYO

To follow the fate of HSCs *in vivo* in spatio-temporal manner during mouse development we produced mouse lines expressing Tamoxifen-inducible Cre recombinase under control of *Sca-1* regulatory elements. These regulatory elements are constructed as a 14kb genomic *Ly-6A* expression cassette containing a distal 3' fragment with strong DNase I hypersensitive sites previously shown to direct high level *in vivo* expression in HSCs (Ma et al., 2001). Briefly, the *CreERT* fusion construct, containing a mutated (G525R) LBD of murine estrogen receptor, was inserted into *ClaI* restriction site of *Ly-6A* gene in the first untranslated exon (**Fig.1**). Three independent *Ly-6A CreERT* transgenic mouse lines were obtained: A, B and C.

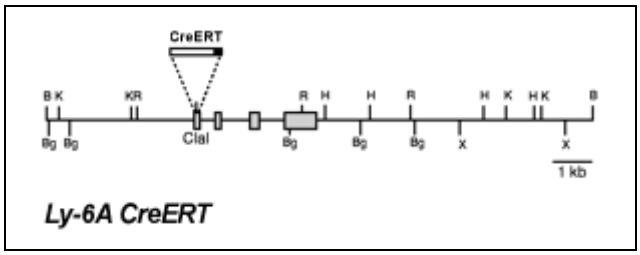

Figure 1. Generation of Ly-6A CreERT transgenic mice. A schematic of *Ly-6A CreERT* construct generated by insertion *CreERT* fragment into *ClaI* site of 14kb *Ly-6A* expression cassette. Restriction sites within the construct include. B -*BamHI*; Bg -*BglII*; K -*Kpn1*; R -*EcoRI*; H -*HindIII* and X -*XbaI*.

To examine whether the *Ly-6A CreERT* transgene was expressed in the midgestation HSC-containing tissues of the 3 transgenic lines, RT-PCR was performed on cDNAs generated from E11 AGM and E11 fetal liver cells. Only one transgenic line, *Ly-6A CreERT* B was found to express Cre (**Fig.2)**. Expression of Cre in Line B was as high as the Cre expression from in the adult bone marrow of the CAG Cre transgenic line (Sakai and Miyazaki, 1997).

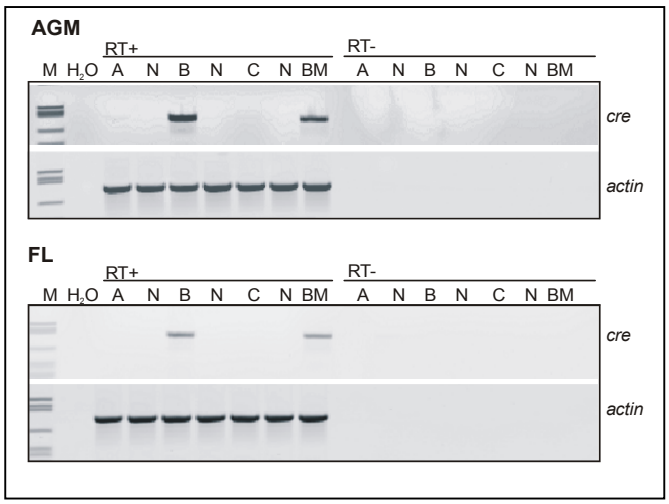

Figure 2. RT-PCR expression analysis of *Ly-6A CreERT* in E11 AGM and fetal liver (FL) of 3 transgenic mouse lines. cDNA was prepared from E11 AGM and FL total RNA (1µg) of 2 individuals of the *Ly-6A CreERT* transgenic lines A,B and C. Lanes marked N denote non-transgenic littermate cDNA. For semi-quantitative measurement of *Ly-6A CreERT* expression *β-actin* normalization was included. RT+, reverse transcriptase added to reaction. RT-, no reverse transcriptase added to reaction. M = size marker (λ/*Pst1*). Bone marrow (BM) from a CAGCre transgenic mouse was used as a positive control.

Line B embryos were also examined for *Ly-6A CreERT* expression in several other tissues (**Fig.3**). Cre was found to be highly expressed in the dissected AGM subregions, dorsal aorta and urogenital ridges. High levels of expression were found also in the vitelline and umbilical vessels. Yolk sac expressed only low levels of Cre. And, as shown in **Figure 4,** Cre is also expressed in the placenta. Thus, Line B *Ly-6A CreERT* transgenic embryos express the transgene in the expected tissue-specific

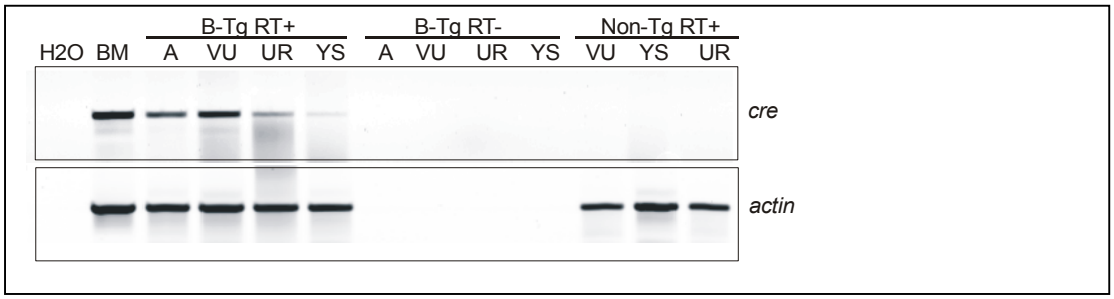

Figure 3. Expression of *Cre* in E11 *Ly-6A CreERT* Line B hematopoietic tissues.
Hematopoietic tissues, including the AGM region and subregions (dorsal aorta and urogenital ridges) were dissected, RNA isolated and cDNAs generated for RT-PCR analysis. Non-transgenic (non-Tg) tissues were used as controls. RT+, reverse transcriptase added to reaction. RT-, no reverse transcriptase added to reaction. BM = bone marrow obtained from CAG Cre adult mouse. A = dorsal aorta; VU = vitteline and umbilical vessels; UR = urogenital ridges and YS = yolk sac.

pattern.

Line B was used for all the following described fate mapping experiments together with two established reporter lines, Rosa26 lox lacZ (Soriano et al., 1999) and Rosa26 lox EGFP (Mao et al., 2001). Upon Cre-mediated recombination these mice will express the β-galactosidase and EGFP reporters under the transcriptional control of the *Rosa26* gene. The *Rosa26* locus is known to be constitutively active during mouse development and is more accessible for site-specific recombination than other genetic loci (Soriano, 1999; Mao et al., 2001; Sohal et al., 2001; Kisseberth et al., 1999).

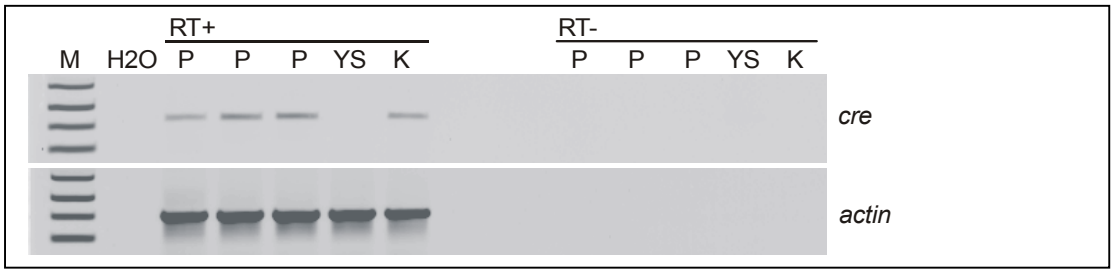

Figure 4. Expression of *Cre* in E11.5 *Ly-6A CreERT* Line B placenta.
cDNA was made from total RNA (1µg) isolated separately from 3 individual Line B transgenic placentas. For semi-quantitative analysis of Ly-6A CreERT expression, β-actin levels were normalized. RT- controls are also included. M = size marker (1kb+ DNA ladder; Invitrogen); YS = yolk sac from non-transgenic control; K = kidney from CAG Cre positive control.

TAMOXIFEN-INDUCIBLE RECOMBINATION IN MURINE EMBRYONIC HEMATOPOIETIC SITES

Ly-6A CreERT induces only rare recombination events in vitro in embryonic hematopoietic tissues

To investigate whether the Ly-6A CreERT transgene mediates recombination *in vitro* following induction by 4OHT, we used a tissue explant culture system that allows for the expansion of the first adult repopulating Sca-1$^+$ hematopoietic stem cells (HSCs) (Medvinsky and Dzierzak, 1996; de Bruijn et al., 2000). After 3 days of culture at the air-medium interface in the presence of 4OHT (5μM), E10 and E11 YS, AGM, FL and body remnant explants obtained from Ly-6A CreERT: Rosa26 lox lacZ embryos were examined histologically for β-galactosidase activity (**Fig. 5**).

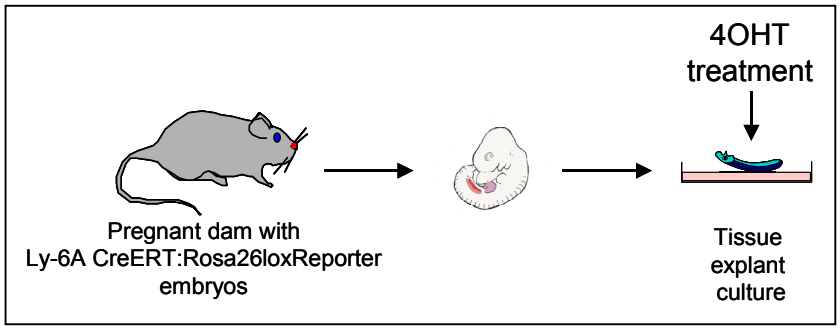

Figure 5. Schematic of procedure used to induce recombination/marking. *Ly-6A CreERT* x *Rosa26 lox LacZ* matings were established and E10/11 AGMs isolated and explant cultured with 4OHT for 3 days.

In our first studies, histological analysis of the sections obtained from E10 explants (32-37sp; n=2) showed no β-galactosidase activity. E11 (44-49sp; n=6) tissue sections also showed no β-galactosidase activity. Although attempts to optimize the staining method (increased the incubation temperature to 37ºC) improved our ability to detect low-level lacZ expression in control samples, we still observed no β-galactosidase positive cells in the organ explants.

The lack of recombination/reporter expression prompted us to change the explant culture conditions. In other studies in the laboratory, IL-3 has been shown to have a significant positive effect on HSC survival and proliferation in E11 AGM explants (C. Robin et al., submitted). *In vitro* explant cultures of E11 Ly-6A CreERT: Rosa26 lox lacZ tissues (YS, AGM, FL and vitelline and umbilical vessels) with IL-3 (100ng/ml) in the presence or absence of 4OHT (1μM) (n=3) were examined for lacZ expressing cells (**Fig. 6)**. A few β-galactosidase positive cells were found in the AGM region around the vessels and in tubular structures, as well as in the vitelline and umbilical arteries. E11 FL and yolk sac control sections showed high background staining (*not shown*) and thus, it could not be determined whether these tissues contained cells in which a recombination had occurred.

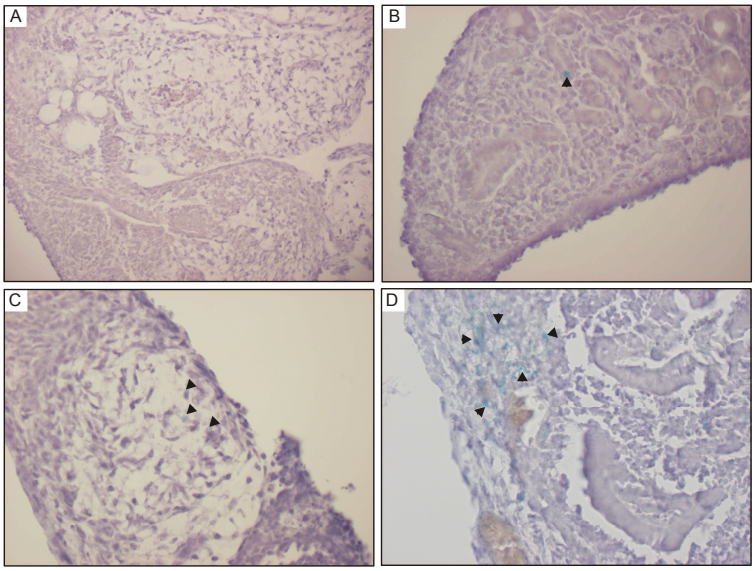

Figure 6. Histological analysis of lacZ expression in E11 hematopoietic tissue explants. E11 vitteline/umbilical vessels with part of the gut (**A, C, D**) and E11 AGM (**B**) from Ly-6A CreERT: Rosa26 lox lacZ embryos were explant cultured for 3 days in the absence (**A**) or presence (**B, C, D**) of 4OHT to induce recombination. After culture all tissues were stained for lacZ activity. Arrows indicate lacZ positive cells in mesonephric tubule (**B**) and in endothelium (**C**) and patches (**D**). Original magnifications are 40x for (**B**, **C**, **D**), 20x for (**A**).

It is known that different lox reporter lines vary in their ability to affect recombination, most likely due to the variance in the genomic positions of and distance between loxP sites (Vooijs et al., 2001; Zheng et al., 2000). Moreover, it has been suggested that reporter gene expression decreases with the age of the animal or that some reporters are lethal to certain lineages of cells (Gilchrist et al., 2003; Kisseberth et al., 1999). Thus, we used another lox reporter line in our studies. Because of the easy visualization of the GFP marker (with no additional staining steps or unwanted background staining) we used the Rosa26 lox EGFP line.

We tested for the recombination/expression of GFP in E11 Ly-6A CreERT: Rosa26 lox EGFP transgenic tissues after explant culture with 4OHT and IL-3. As a positive control to confirm our ability to detect GFP expression in such cultured tissues, we examined cryosections of E11 yolk sac explants from Ly-6A GFP transgenic embryos. Consistent with previous studies (de Bruijn et al., 2002), we found GFP expressing hematopoietic cells in some vessels as well as GFP expressing endothelial cells in the yolk sac (**Figs. 7B** and **F**). However, no GFP positive cells were found in cryosections of E11 yolk sac and E11 AGM double transgenic 4OHT-treated explants (**Figs. 7D** and **H**). Yolk sac showed only high autofluorescence. Thus, the lack of GFP expression in E11 4OHT-treated tissues suggests that either no Cre-mediated recombination took place or GFP is expressed at very low levels or in very rare cells.

To be able to perform the analysis of GFP expression in more sensitive and quantitative manner, E11 Ly-6A CreERT: Rosa26 lox EGFP transgenic tissues (yolk sac, fetal liver and AGM+vitelline/umbilical+gut) after explant culture in the absence or presence of 4OHT were dispersed into a single-cell suspension and analyzed for expression of the recombination marker, GFP by flow cytometry (FACScan; BD

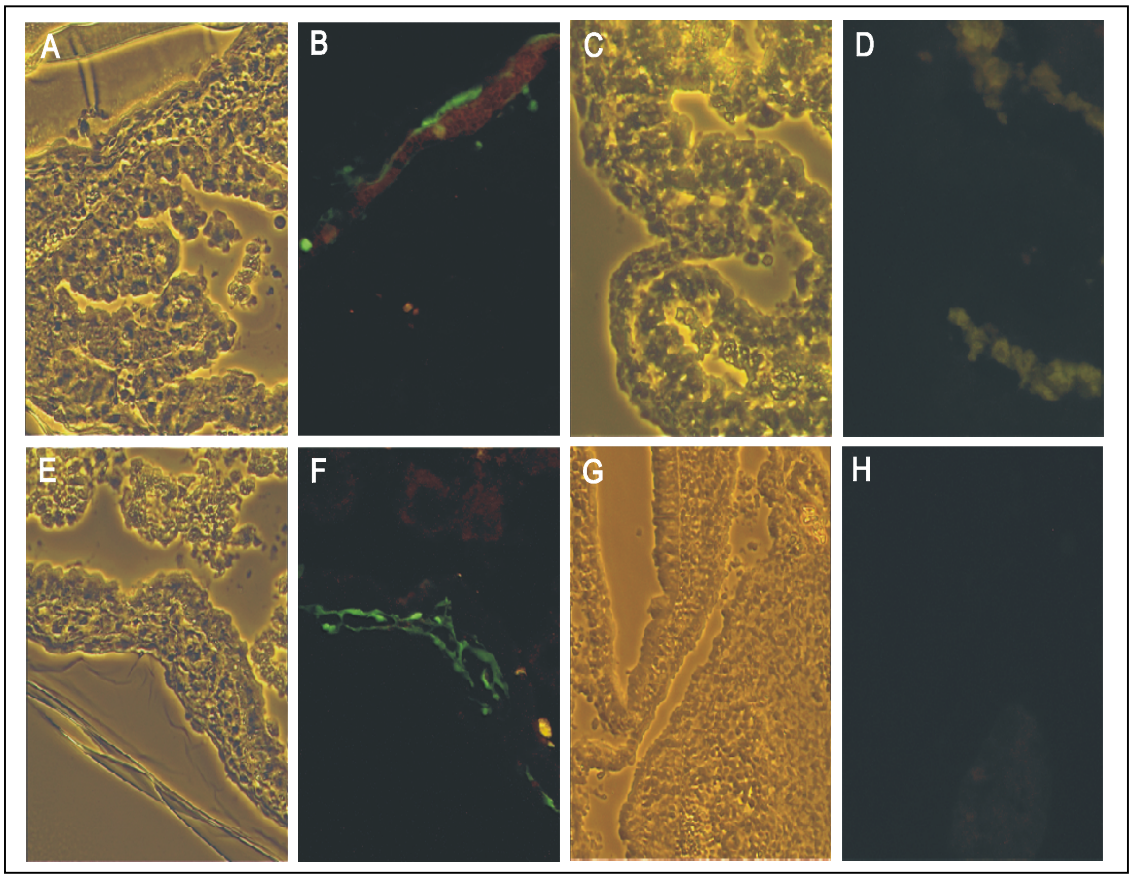

Figure 7. Analysis of GFP expression in E11 tissues after the explant culture.
Microscopic bright field (**A, C, E, G**) and corresponding GFP fluorescent (**B, D, F, H**) images of cryosectioned explants. **A**, **B**, **E**, **F** are sections from Ly-6A GFP transgenic E11 YS explants. **C** and **D** are sections from Ly-6A CreERT: Rosa26 lox EGFP E11 YS explant and **G** and **H** are sections from a Ly-6A CreERT: Rosa26 lox EGFP E11 AGM explant. Original magnification for the images: 10x or 20x.

Biosciences). Unfortunately, no GFP positive cells were found (**Fig.8**).

To ensure that we were not missing rare GFP expressing cells, we performed sensitive *in vivo* transplantation experiments. We postulated that recombined HSCs may be better observed after engraftment, amplification and differentiation in an irradiated adult host. We coinjected cells from AGM+vitelline/umbilical+gut explants treated with 4OHT (<1 tissue transplanted per recipient), together with adult spleen cells ($2x10^5$; to promote survival of recipients), into adult irradiated recipients. At 1 month post transplantation we analyzed DNA isolated from peripheral blood (PB) of the transplanted mice by semi-quantitative GFP PCR. Indeed, 8 out of 20 mice were engrafted with donor cells but no GFP expressing cells were found in PB of any of these

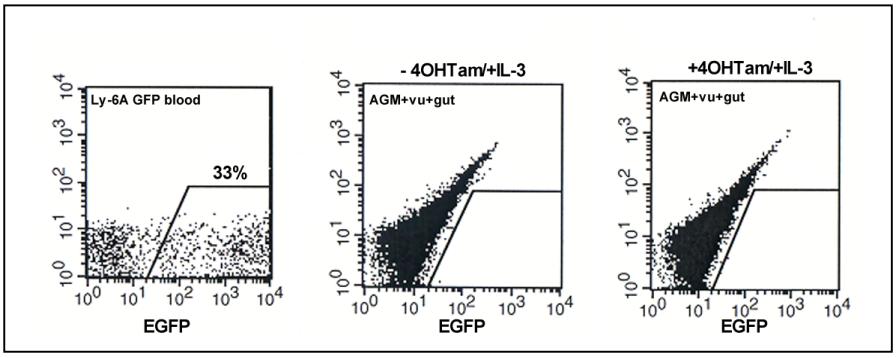

Figure 8. Flow cytometric analysis for expression of recombination marker GFP in E11 AGM explants. E11 Ly-6A CreERT: Rosa26 lox EGFP transgenic tissues were explant cultured with IL-3 in the absence or presence of 4OHT for 3 days. Cells were harvested and analyzed for GFP expression by flow cytometry. As a positive GFP control, peripheral blood from adult Ly-6A GFP was analyzed.

8 engrafted mice (data not shown). We further analyzed the hematopoietic organs and peripheral blood after 4 months post transplantation 2 of the 8 engrafted recipients. Although these 2 recipients were high-level, multilineage repopulated by donor cells in all hematopoietic tissues, no GFP positive cells were detected by flow cytometric analysis. Thus, these data strongly suggest that no or only rare undetectable recombination events occurred in Rosa26 lox EGFP HSCs during the AGM explant culture. Taken together with the results of the lox lacZ reporter embryos, only rare recombination events can be induced *in vitro* in midgestation CreERT: loxP reporter transgenic hematopoietic tissues in the presence of 4OHT.

Tamoxifen-inducible recombination in vivo

To trace embryonic hematopoietic cells and HSCs during ontogeny we performed *in vivo* recombination experiments in which pregnant dams (from matings between Ly-6A CreERT males and Rosa26 lox reporter females) were treated with Tamoxifen. Previous studies suggested that a single intraperitoneal (i.p.) injection of 1mg of Tamoxifen at E9.5 or later results in rapid and effective recombination with a minimum of embryonic lethality, while injection of 2 mg of Tamoxifen leads to very high embryonic lethality at term (Danielian et al., 1998). Since Tamoxifen is an anti-estrogen drug, it may interfere with the maintenance of a pregnancy at high doses (White, 1999). In contrast, other reports have shown that a single administration of higher dose of Tamoxifen, 3mg at E8.5 to Rosa26 lox lacZ pregnant females, has no effect on the proper development of most embryos (Hayashi and McMahon, 2002). Moreover, injection of Tamoxifen at the 3mg dose results in high efficiency of Cre-mediated recombination, followed by activation of reporter gene expression within 24 hours. Hence, we performed a series of experiments that included injecting pregnant dams i.p. with varying doses and dosage regimens of Tamoxifen at 9, 10, 11, 13 and/or 14 days post coitum. The protocol for these studies is shown in **Figure 9**.

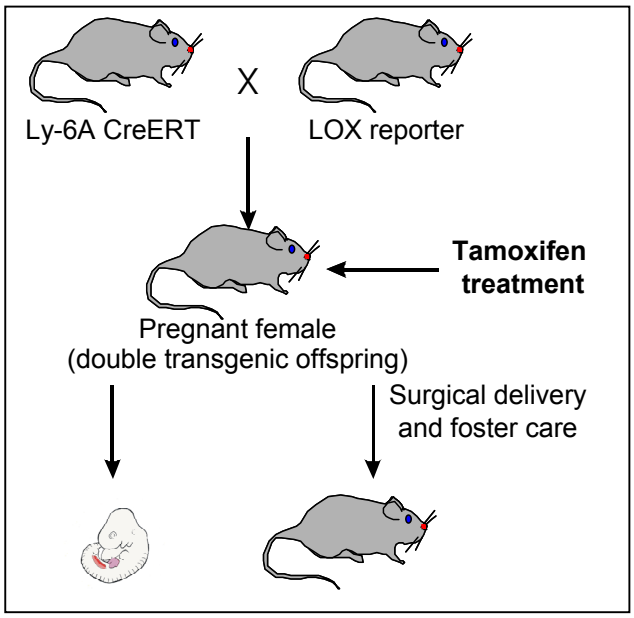

Figure 9. Schematic of matings and *in utero* Tamoxifen treatment of offspring examined in fate mapping experiments.
Ly-6A CreERT x *Rosa26 lox reporter* matings were established and pregnant dams were intraperitoneally injected with Tamoxifen to induce genetic marking of HSCs at E9-E14. Embryos, neonates and adult offspring were analyzed for expression of the recombined reporter.

Tamoxifen-inducible recombination/expression in vivo after a single Tamoxifen injection – embryo analysis

In our first experiments, day 9 post coitum Rosa26 lox lacZ pregnant females (mated with Ly-6A CreERT males) were injected i.p. one time with 2mg of Tamoxifen. We scored double transgenic (Ly-6A CreERT: Rosa26 lox lacZ) embryos and single transgenic control embryos at E13 or E14. Several hematopoietic tissues were dissected, processed and examined by lacZ staining to detect Tamoxifen-induced recombination/expression (*not shown*). Microscopic analysis of E14 double transgenic tissues sections showed only very few lacZ positive cells in the yolk sac. LacZ expression was also detected in E13 double transgenic metanephros, but only within a very low number of cells. These rare positive cells were not found in the single transgenic control tissue sections.

We next injected day 9 post coitum Rosa26 lox lacZ pregnant females i.p. (mated with Ly-6A CreERT males) with a single 3mg dose of Tamoxifen. Histological analysis of lacZ stained whole-mount and tissue sections of E13 double transgenic (Ly-6A CreERT: Rosa26 lox lacZ) embryos (n=11) treated *in utero* with Tamoxifen showed lacZ expression in the yolk sac and AGM, suggesting that recombination occurred in these hematopoietic tissues (**Fig. 10**). While no lacZ positive cells were found in the control yolk sac and AGM sections, a low level of lacZ staining was observed in the gut region of control E13 double transgenic embryos (n=8) treated *in utero* with the oil (the vehicle for Tamoxifen delivery). Unfortunately, lacZ expressing cells were not found in E13 or E14 fetal liver, a previously demonstrated site of Ly-6A expression. Thus, with the exception of the fetal liver, the Ly-6A CreERT recombination/expression present in

the yolk sac and AGM region is consistent with the expression pattern expected based

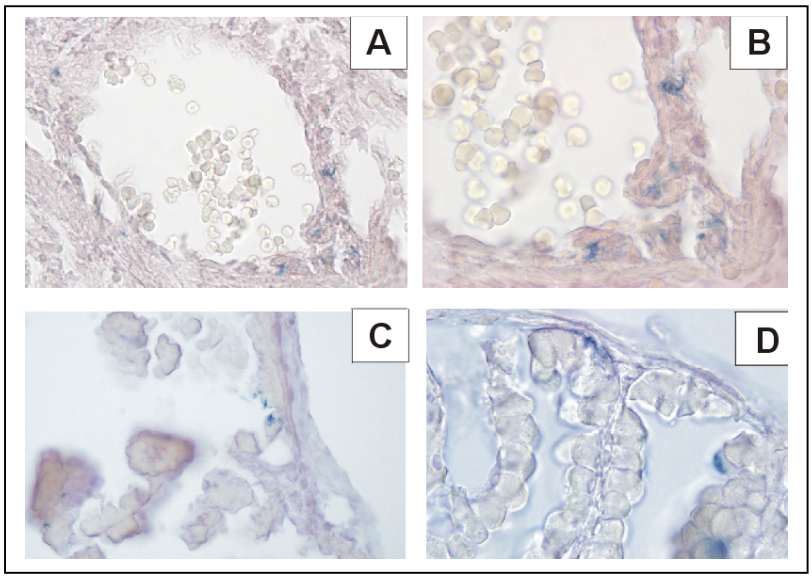

Figure 10. Histological analysis of lacZ expression in E13 AGM and E13 YS of an embryo treated *in utero* with Tamoxifen at E9. Microscopic image of transverse section of the dorsal aorta in AGM region at 20x **(A)** and **(B)** 100x magnifications. LacZ positive cells are within the ventral side of the aorta in the endothelium/mesenchyme. (**C**, **D**) lacZ expressing cells are detected in E13 YS at 40x **(C)** and 100x **(D)** magnifications.

on studies of Ly-6A GFP transgenic mice (de Bruijn et al., 2002).

In order to increase the level of recombination product observed in E13 hematopoietic tissues, we injected 4mg of Tamoxifen into day 9 post coitum Rosa26 lox lacZ females (mated with Ly-6A CreERT males) and performed lacZ staining at 37ºC. We again detected a few lacZ positive cells within E13 yolk sac and AGM region, but did not detect any lacZ positive cells in the fetal liver at this stage (*not shown*), thus confirming our previous result.

Tamoxifen-inducible recombination/expression in vivo after two Tamoxifen injections – embryo analysis

Recently published data (Göthert et al., 2005) has shown consistent recombination in E14 FL of double transgenic embryos (SCL-CreERT: Rosa26 lox EYFP) obtained from pregnant females injected i.p. on 2 consecutive days with Tamoxifen; 1mg on E10.5 and 2mg on E11.5. The percentage of recombined, YFP positive cells was 4% of total E14.5 fetal liver cells. Further depletion of differentiated cells and HSC enrichment by flow cytometry using HSC markers resulted in an increased percentage (17%) of YFP expressing FL cells within KSL (c-kit$^+$Sca-1$^+$Lin$^-$) population, indicating that hematopoietic stem/progenitor cells from E14.5 fetal liver were efficiently marked.

Following a similar temporal Tamoxifen injection protocol (E10.5 and E11.5), but with an increased dose of Tamoxifen (4mg/day), we analyzed Ly-6A CreERT-mediated recombination/lacZ expression in E14 fetal liver cells (n=11) obtained from compound embryos (Ly-6A CreERT: Rosa26 lox lacZ) receiving two *in utero* injections. In order to detect lacZ positive cells we performed FDG staining and flow cytometric analysis

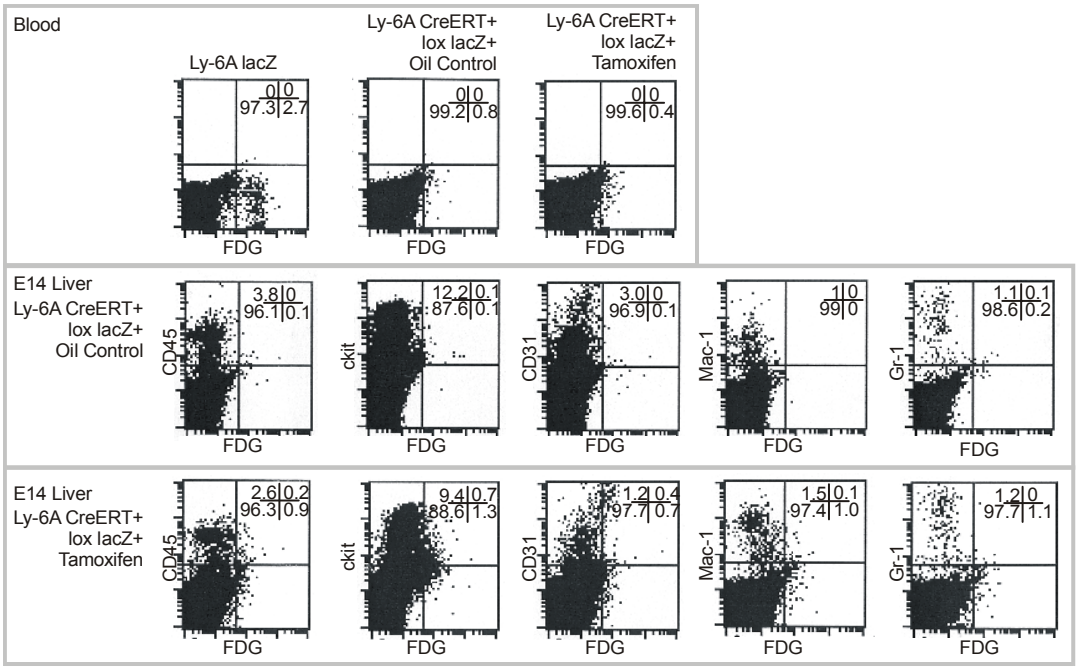

Figure 11. Flow cytometric analysis for coexpression of lacZ and hematopoietic markers on E14 blood and fetal liver (FL) cells from Ly-6A CreERT: Rosa26 lox lacZ embryos treated *in utero* (E10.5 and E11.5) with 4 mg of Tamoxifen or oil. FL was made into a single cell suspension and stained with FDG (after permeabilization) and antibodies specific for CD45, c-kit, CD31, Mac-1 and Gr-1. Blood from a Ly-6A lacZ transgenic adult was the positive lacZ control. Percentages of cells in each quadrant are shown in the upper right corner of each plot.

(**Fig. 11**). We included Ly-6A lacZ transgenic blood as a positive control. *In utero* oil treated E14 double transgenic embryos were used as a negative control. Blood taken from the oil controls showed 0.8% FDG positive cells, whereas the blood from Tamoxifen treated embryos showed only 0.4% FDG positive cells. This suggests that the Tamoxifen treatment did not induce recombination/expression, at least in circulating blood cells. In contrast, FL did show an increase in FDG positive cells in the Tamoxifen treated embryos. Oil control E14 FL (n=3) showed 0.1 to 0.3% FDG positive cells, while E14 FL from *in utero* Tamoxifen-treated embryos showed 1.1 to 2.0% FDG positive cells. A fraction of the FDG positive cells were hematopoietic, as shown by staining with CD45, c-kit and Mac-1 specific antibodies. Also, some of the FDG positive E14 FL cells could be endothelial as determined by high expression of the CD31 marker (0% in oil-treated versus 49% in Tamoxifen-treated embryos). Thus,

some small fraction of fetal liver cells from E10/E11 Tamoxifen-treated embryos express the lacZ recombination marker.

Since the GFP marker does not require an antibody staining step and has lower background, we next performed the same two-injection *in utero* treatment experiment using the Rosa26 lox EGFP marker transgenic line. Ly-6A CreERT: Rosa26 lox EGFP embryos were treated *in utero* with 4 mg of Tamoxifen at E10.5 and another 4 mg of Tamoxifen at E11.5. Flow cytometric analysis was performed on E13/14 fetal liver cells for GFP expression. In contrast to the experiments using the Rosa lox lacZ reporter, no background fluorescence was observed in E13/14 control fetal liver. However, no Tamoxifen induced recombination/expression of GFP was found in the compound transgenic E13/14 fetal liver (*not shown*). Thus, there is either no recombination induced in the Rosa26 lox EGFP transgene or the recombination events are so rare that they are not detectable in the small number of mice analyzed. Alternatively, the transcription of EGFP could be so low that threshold fluorescence detection levels of the protein are not achieved (*see next section for data on this point*). Taken together, the data obtained from histological and flow cytometric analysis of expression of the lacZ recombination marker in different hematopoietic tissues at E13 and E14 suggest that recombination driven by Ly-6A creERT transgene occurred, at best, in only rare cells in hematopoietic tissues and that events are rare even when two injections of high dose Tamoxifen are administered *in utero*.

Do genetically marked embryonic hematopoietic (stem) cells contribute to adult hematopoiesis?

The direct relationship between embryonic and adult hematopoietic (stem) cells remains still not well understood. Recent data obtained by Göthert et al. have shown that approximately 10% of adult HSCs originate from embryonic HSCs, which were genetically marked by Cre-mediated recombination induced at E10.5/E11.5 (Göthert et al., 2004). However, these studies did not specifically mark AGM HSCs. Thus, it is still unclear if adult type of HSCs generated autonomously in the AGM region at E10.5 during normal developmental processes are the founder cells for the adult hematopoietic system. In an attempt to mark hematopoietic (stem) cells in the AGM region and trace them to the adult stage, we performed fate mapping experiments with reporter lines Rosa26 lox lacZ and Rosa26 lox EGFP and the Ly-6A CreERT transgenic line.

Examination of neonates treated *in utero*.

In order to trace recombined embryonic hematopoietic cells to the neonatal stage, we perform initially a single i.p. injection of 3mg of Tamoxifen at E9 into pregnant Rosa26 lox lacZ females (mated with Ly-6A CreERT males), followed by analysis of different hematopoietic neonatal organs at E18. In the first experiment, lacZ positive cells were found in serial sections of the bone marrow within the femur (**Fig.12**) and some cells within the thymus (*not shown*), suggesting that the Cre-mediated recombination occurred within the E9 AGM region. This implies that cells

marked by Cre-mediated recombination in the AGM region or their progeny, migrated and colonized the neonatal hematopoietic organs. Unfortunately, we had no E18 controls (non-transgenic or oil-treated) available at the time of this staining. Hence, we cannot rule out that the possibility that the observed staining is only high background.

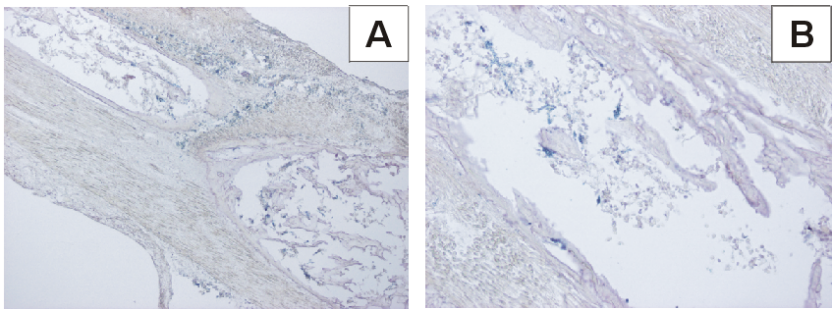

Figure 12. Histological analysis of lacZ expression in E18 bone marrow of a mouse treated *in utero* with Tamoxifen at E9. Microscopic image of neonatal bone marrow with different magnifications; **A**.is 20x and **B** is 40x.

We continued with these experiments and attempted to induce recombination at a slightly later developmental time (E13/E14) in the FL. At this time FL contains large numbers of HSCs and thus, we hoped to increase our chances of recombination/expression events. Rosa26 lox lacZ pregnant females (mated with Ly-6A CreERT males) were treated with 4mg of Tamoxifen on each of 2 consecutive days, E13 and E14. The first litter of mice obtained was examined by FDG-flow cytometry. 0.95% of E18 liver cells from double transgenic (Ly-6A CreERT: Rosa26 lox lacZ) neonates (n=4) were FDG positive. Analysis of bone marrow pooled from the 4 neonates showed that 0.34% of the bone marrow cells expressed lacZ. However, no age-matched non/single-transgenic or oil-injected controls were available at the time of the analysis, thus, we could not make any firm conclusions concerning recombination/expression.

In another experiment, FDG flow cytometry was used to analyze cells from various hematopoietic tissues of a Ly-6A CreERT: Rosa26 lox lacZ neonate (treated *in utero* with 4mg Tamoxifen at E13 and E14) and a control wild type neonate. Ly-6A lacZ transgenic blood was used as a positive control. Several hematopoietic subpopulations were examined to determine, in the case of lacZ expression, if these cells were hematopoietic. As shown in **Figure 13**, although FDG positive cells were found in the bone marrow of the double transgenic *in utero* Tamoxifen-treated neonate, the percentage of these cells was not above the background seen in the wild type animal. Moreover, the FDG positive fractions of the spleen and liver of the test animal also did not exceed the percentage that is characteristic of background staining.

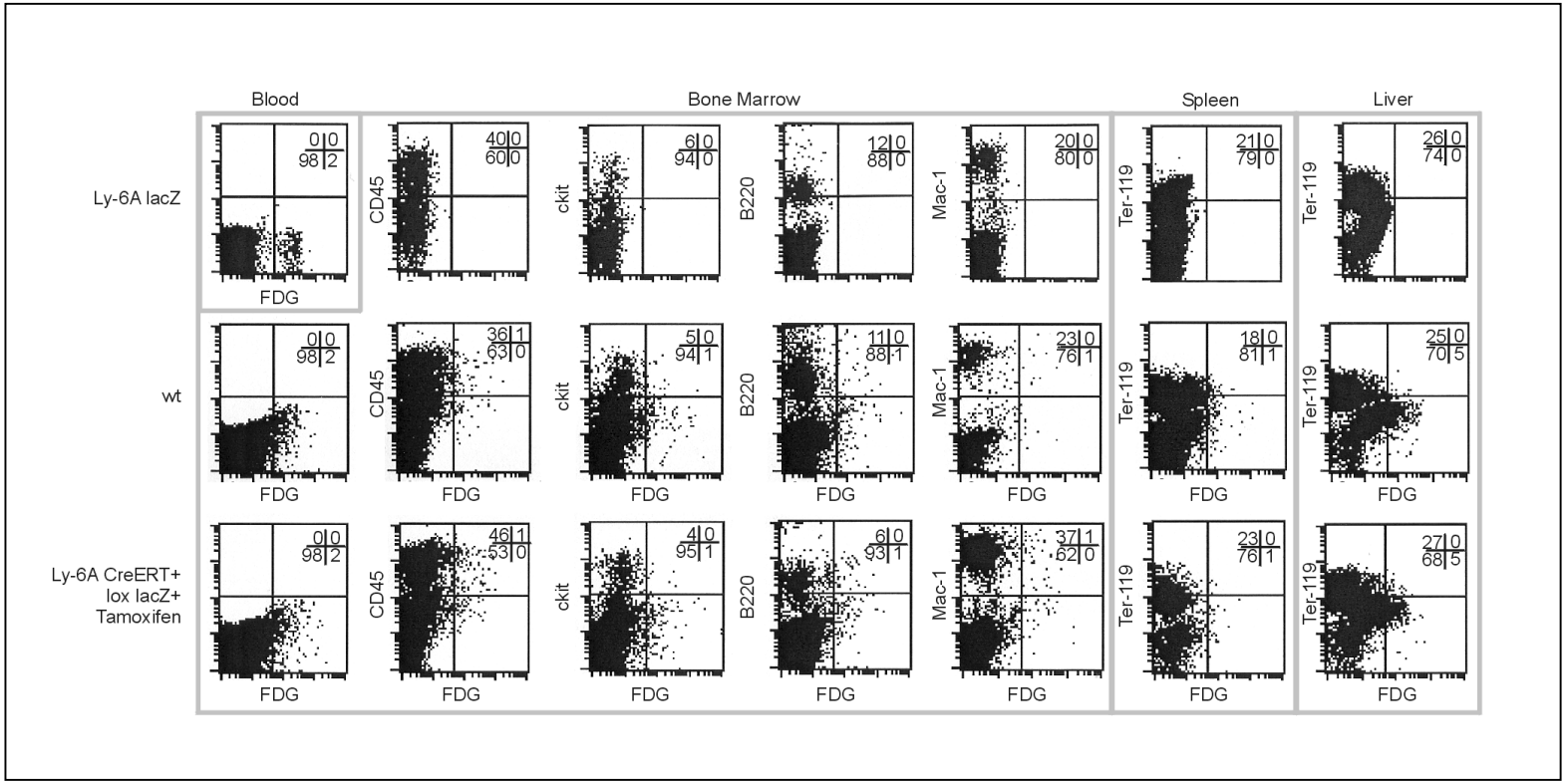

Figure 13. Flow cytometric analysis for coexpression of lacZ and hematopoietic markers on cells from Ly-6A CreERT: Rosa26 lox lacZ neonates treated *in utero* (E13 and E14) with 4 mg of Tamoxifen.

Hematopoietic tissues (bone marrow, spleen and liver) were harvested on E18. Single cell suspensions were stained with FDG (after permeabilization) and antibodies specific for CD45, c-kit, B220, Mac-1 and Ter-1119. Blood from a Ly-6A lacZ transgenic adult was the positive lacZ control. A wild type neonate provided cells used as the single staining (positive) and double staining (negative) controls. Percentages of cells in each quadrant are shown in the upper right corner of each plot. To note, similar results were obtained by histogram analysis (*not shown*).

We also analyzed Ly-6A CreERT: Rosa26 lox GFP double transgenic and single

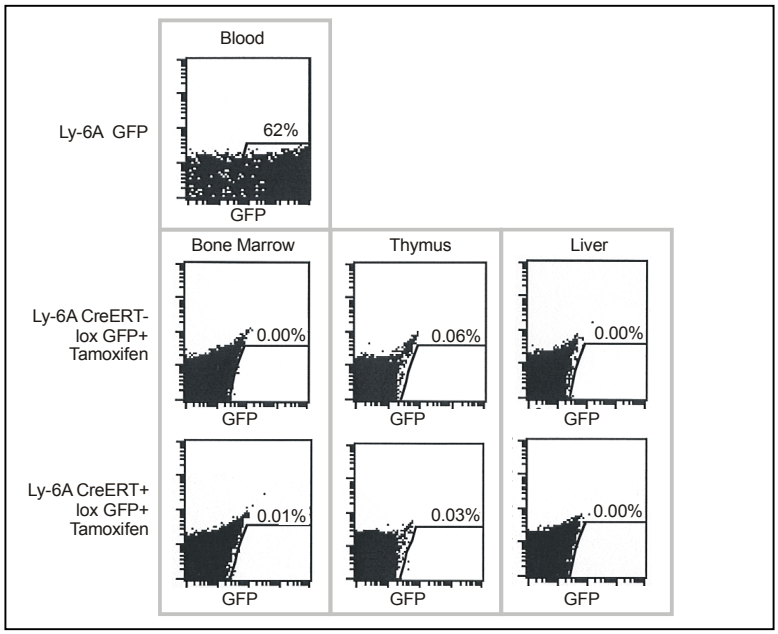

Figure 14. Flow cytometric analysis for expression of GFP on cells from Ly-6A CreERT: Rosa26 lox EGFP neonates treated in utero (E13 and E14) with 4 mg of Tamoxifen. Bone marrow, thymus and liver were harvested on E18 and single cell suspensions were examined for GFP fluorescence. Blood from a Ly-6A GFP transgenic adult was the positive GFP control. An *in utero* treated Rosa26 lox EGFP neonate served as a negative control. Percentages of cells in gated region are shown.

transgenic neonatal animals that were *in utero* treated with 4mg Tamoxifen on E13 and E14 (**Fig. 14**). We examined bone marrow, thymus and liver cells by flow cytometry for GFP. Ly-6A GFP adult blood served as a positive control. In both, the control single transgenic and the double transgenic neonates, no GFP positive cells were detected.

Examination of adults treated *in utero*.

Our studies next focused on the examination of adult offspring that had been treated *in utero* with Tamoxifen. We had previously observed that pregnant dams injected with Tamoxifen had difficulties in delivery or were completely unable to deliver their pups. To ensure that adult offspring were obtained, we performed surgical deliveries at 18 days post coitum, just before the time expected for natural parturition. After surgical delivery, the pups were fostered by lactating wild type (FVB) females.

Double transgenic (Ly-6A CreERT: Rosa26 lox lacZ) adult mice (n=4) treated *in utero* at E9 with 4mg of Tamoxifen were examined by FDG flow cytometry for lacZ expression. An average of 1.3% of bone marrow cells from such mice were FDG positive as compared to 0.7% FDG positive bone marrow cells from negative control adult mice (Ly-6A CreERT single transgenic) treated *in utero* at E9 with Tamoxifen. The double transgenic mouse with the highest percentage of FDG positive BM cells (1.7%) was examined for co-expression of hematopoietic markers such as Sca-1, CD45, c-kit, CD34, CD31, B220 and Mac-1 (**Fig. 15**). Interestingly, most of the FDG positive bone marrow cells in the double transgenic and the negative control were detected in $CD45^+$ population. A slightly higher percentage of FDG^+CD45^+ cells was found in the double transgenic (1.5%) as compared to the negative control (1.1%). Although very

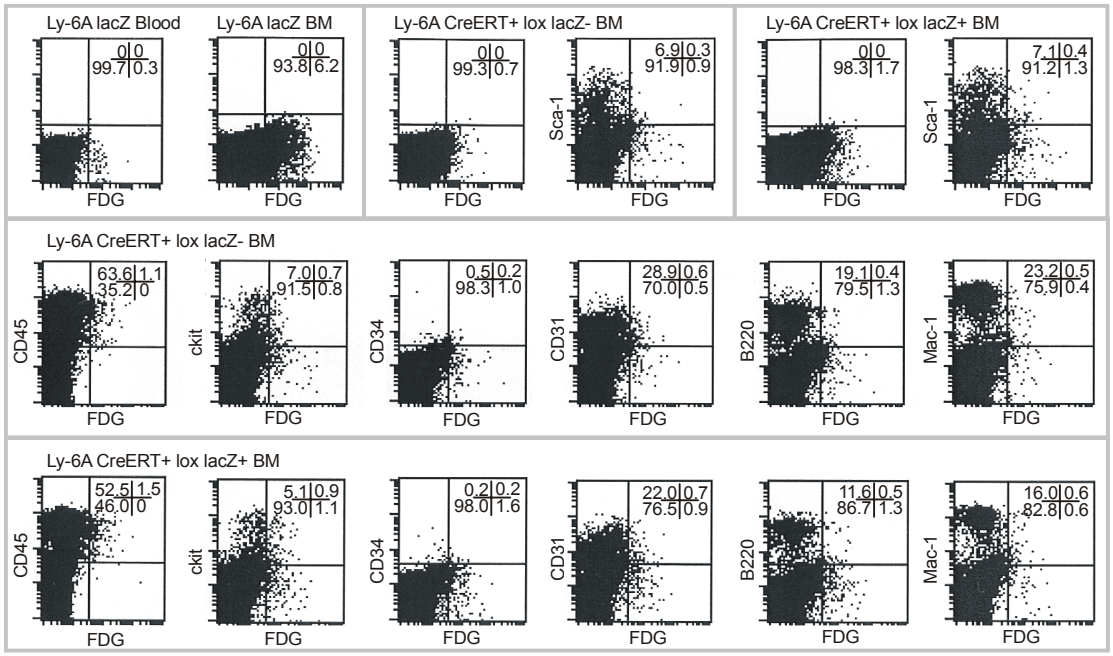

Figure 15. Flow cytometric analysis for coexpression of lacZ and hematopoietic markers on cells from Ly-6A CreERT: Rosa26 lox lacZ adults treated *in utero* at E9 with 4 mg of Tamoxifen. Bone marrow was harvested and single cell suspensions were stained with FDG (after permeabilization) and antibodies specific for Sca-1, CD45, c-kit, CD34, CD31, B220 and Mac-1. Blood from a Ly-6A lacZ transgenic adult served as the positive lacZ control. A Ly-6A CreERT littermate served as the negative control. Percentages of cells in each quadrant are shown in the upper right corner of each plot.

minor, increased percentages were also observed in the $FDG^+Sca\text{-}1^+$, $FDG^+c\text{-}kit^+$, FDG^+CD31^+, FDG^+B220^+ and $FDG^+Mac\text{-}1^+$ populations of the double transgenic bone marrow. Thus, although negative control bone marrow gave an FDG staining background (making it difficult to determine with high accuracy the efficiency of expression of the recombination marker), we found a consistent increase in the percentage of FDG positive cells in Ly-6A CreERT: Rosa26 lox lacZ *in utero* Tamoxifen-treated adult mice.

Since we could detect lacZ positive cells in the BM of double transgenic (Ly-6A CreERT: Rosa26 lox lacZ) adult mice treated with a single *in utero* injection of Tamoxifen, we tested if an increase in the amount of Tamoxifen administered would result in a greater efficiency of the Ly-6A CreERT-mediated recombination/expression.

We analyzed by FDG flow cytometry, adult mice (Ly-6A CreERT: Rosa26 lox lacZ) that had been *in utero* treated with Tamoxifen at E10 (4mg) and E11 (4mg). As shown in **Figure 16**, 2.2% of double transgenic spleen cells were FDG positive, as compared to only 1.4% positive cells in the wild type littermate control. Mostly all of the FDG cells of the double transgenic expressed Sca-1, B220 and Mac-1.

Although the FDG positive cells found in the negative control also express Sca-1, B220 and Mac-1, there was a consistent increase above background in the percentage of FDG positive cells in the double transgenic adult spleen cells expressing these markers (especially in Mac-1^{+high} cell population). Although the exact type of these recombined hematopoietic cells remains unclear, these results suggest that hematopoietic cells marked in the midgestation embryo contribute to the adult hematopoietic system.

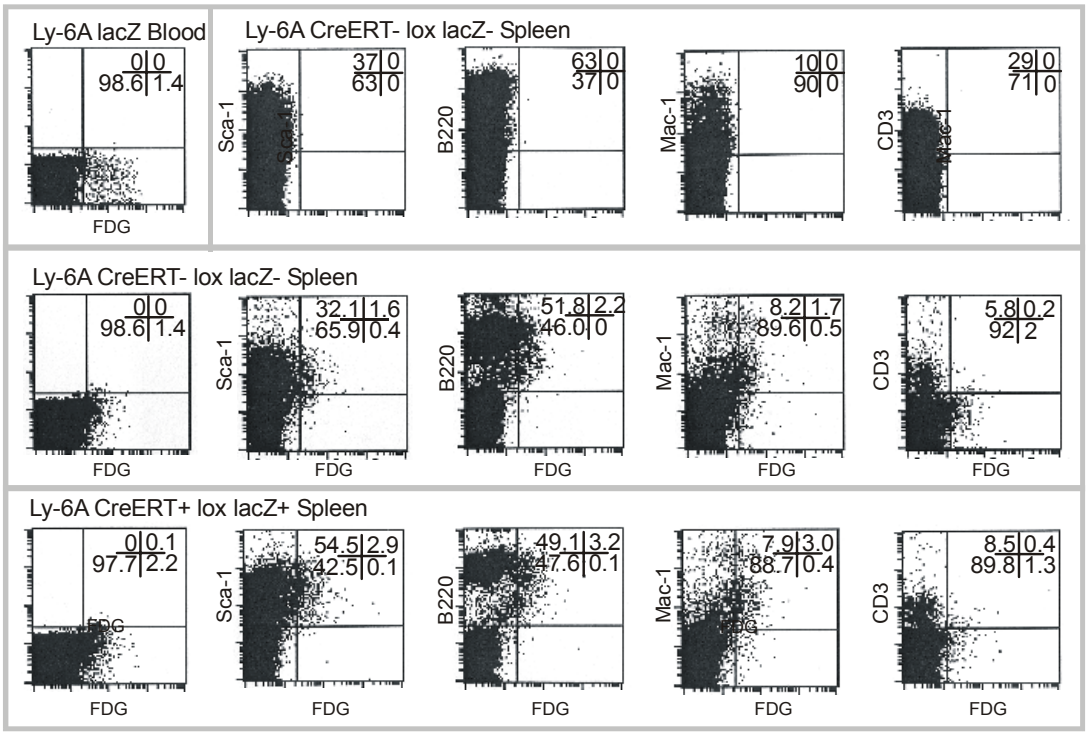

Figure 16. Flow cytometric analysis for coexpression of lacZ and hematopoietic markers on cells from a Ly-6A CreERT: Rosa26 lox lacZ adult and wild type littermate treated *in utero* at E10 and E11 with 4 mg of Tamoxifen. Spleens were harvested and single cell suspensions were stained with FDG (after permeabilization) and antibodies specific for Sca-1, B220, Mac-1 and CD3. Blood from a Ly-6A lacZ transgenic adult served as the positive lacZ control. Control single antibody stainings are shown for wild type spleen. Percentages of cells in each quadrant are shown in upper right corner of each plot.

In attempt to minimize the background, we next performed tracing experiments using Rosa26 lox EGFP reporter line. Treatment of pregnant Rosa26 lox EGFP females (mated with Ly-6A CreERT males) with 4mg of Tamoxifen at day 9 post coitum did not result in detection of any GFP expressing cells within different hematopoietic organs of double transgenic (Ly-6A CreERT: Rosa26 lox EGFP) adult offspring (n=4) compared to a negative adult control mice (n=2) treated *in utero* with oil at E9. The same negative results were obtained when blood, spleen, thymus, lymph node and bone

marrow cells from adult double transgenic mouse (Ly-6A CreERT: Rosa26 lox EGFP) treated *in utero* at E9 and E10 with 4mg of Tamoxifen were analyzed (**Fig.17**).

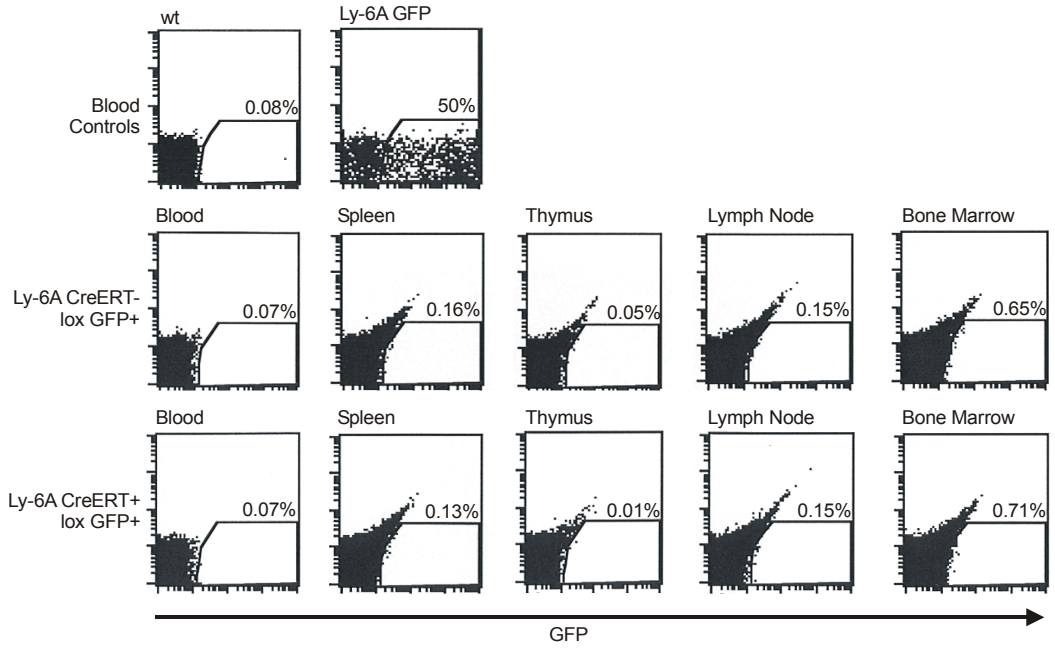

Figure 17. Flow cytometric analysis of GFP expression on cells from Ly-6A CreERT: Rosa26 lox EGFP adults treated *in utero* at E9 and E10 with 4 mg of Tamoxifen. Blood, spleen, thymus, lymph nodes and bone marrow were harvested and single cell suspensions were examined for GFP fluorescence. An *in utero* (E9 and E10) Tamoxifen-treated Rosa26 lox EGFP adult littermate was also examined. Blood from a Ly-6A GFP transgenic adult was the positive GFP control and wild type adult blood was the negative control. Percentages of cells in gated region are shown.

Is Ly-6A CreERT transgene able to induce recombination in vivo within adult hematopoietic system?

Our results from the analysis of Ly-6A CreERT: lox reporter embryos and adults *in utero* Tamoxifen-treated show at best only very rare marked hematopoietic cells. Since Ly-6A (Sca-1) is expressed on adult hematopoietic stem cells as well as many differentiated hematopoietic cell types, we tested whether Tamoxifen treatment of adult double transgenics would lead to a higher efficiency of reporter recombination/expression. Adult Ly-6A CreERT: lox lacZ transgenic mice were treated every 3 or 4 days for 3 times in total with 3-4 mg of Tamoxifen or oil vehicle. The injection schedule is shown in **Figure 18**. FDG flow cytometric analysis of peripheral

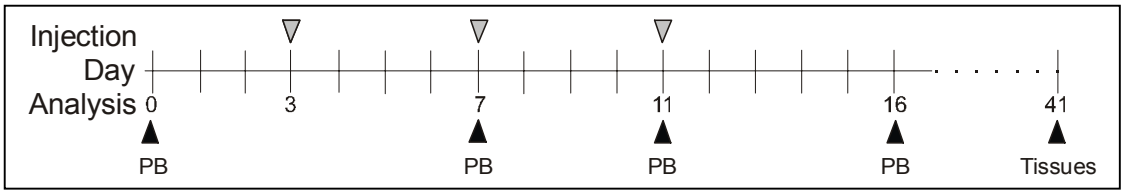

Figure 18. Schematic of Tamoxifen or oil treatment protocol used to induce recombination in adult mice. Adult mice were i.p. injected 3 times with 3 or 4mg of Tamoxifen or oil (grey arrowheads). Before Tamoxifen/oil treatment (day 0) and at day 7, 11 and 16 peripheral blood (PB) was analyzed by FDG-flow cytometry and at times indicated by black arrowheads. Thirty days after the last injection, the mice were sacrificed and all hematopoietic organs were analyzed for lacZ expression by FDG-flow cytometry.

blood at different time points (day 0,7, 11, 16) post-Tamoxifen/oil treatment (n=4) were performed. Following each injection, a small increase (1.6x to 2.7x) in lacZ positive cells was found in double transgenic Tamoxifen-treated adults as compared to oil treated controls. Thirty days following the last injection, we analyzed the hematopoietic organs (lymph node, bone marrow, spleen and thymus) from 2 Tamoxifen- and 2 oil-treated mice for lacZ expressing cells by FDG-flow cytometric analysis. Lymph node and spleen showed a slightly higher percentage of lacZ positive cells in the Tamoxifen

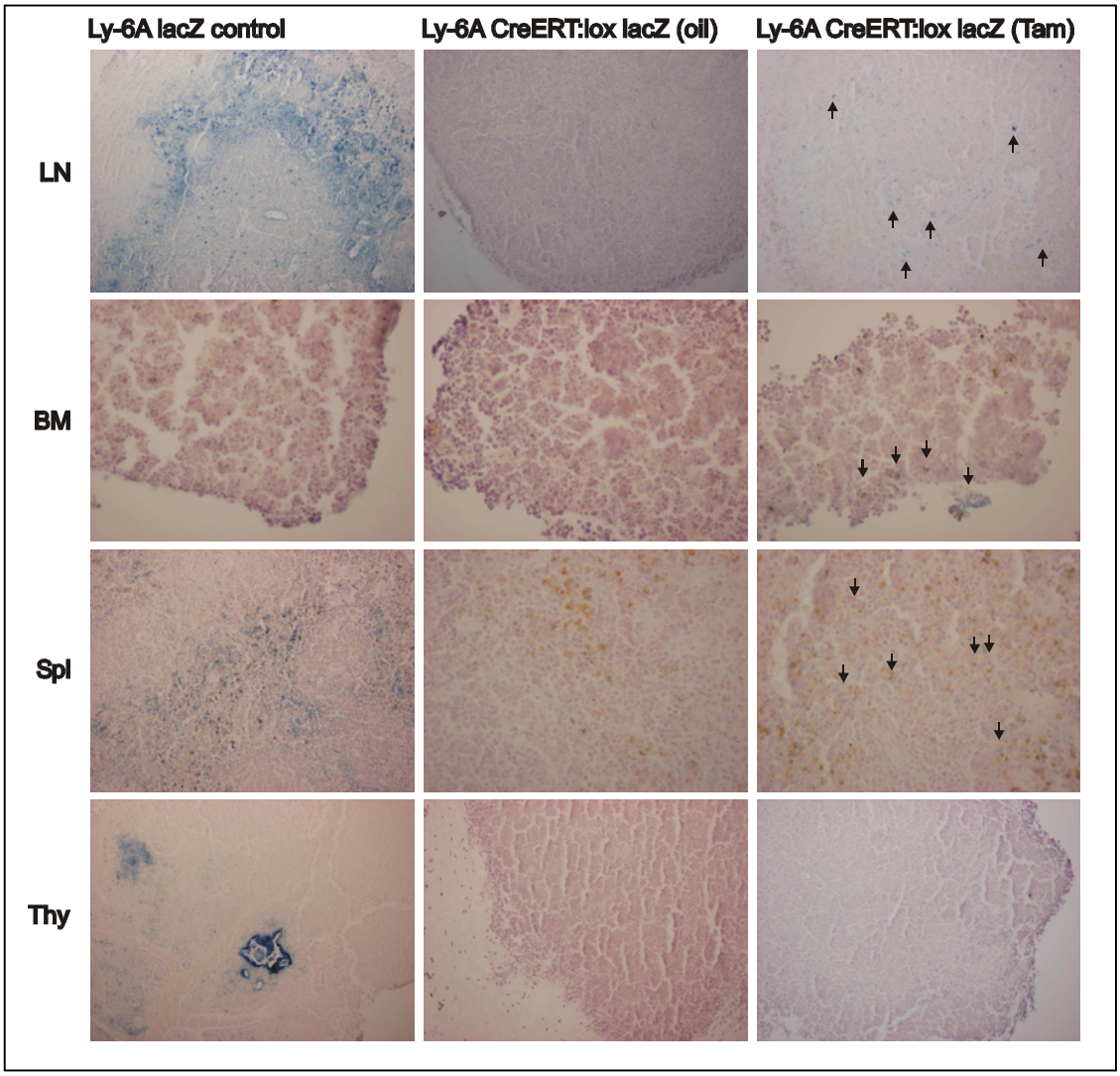

Figure 19. Histological analysis of lacZ expression in the hematopoietic organs isolated from double transgenic adult mice treated with Tamoxifen or oil.
LN (lymph nodes), BM (bone marrow), Spl (spleen) and Thy (thymus) were isolated from adult double transgenic mice (Ly-6AcreERT: Rosa26 lox lacZ), treated either with Tamoxifen (right column) or oil (middle column). Tamoxifen treatment was performed according to the protocol presented in the Fig.18. LacZ stained positive control organs from Ly-6A lacZ adult were included (left column). Hematopoietic organs from oil-treated adult double transgenic mouse were included as negative controls. LacZ positive cells were found in the LN, Spl and BM (some low background in oil-treated mice) but not in the thymus of double transgenic Tamoxifen-treated mice. Black arrows show lacZ positive cells. Original magnifications (left to right) for: LN 10x, 20x and 20x; BM 40x; Spl 20x, 40x, 40x; Thy 10x, 20x, 20x.

treated adults (1.3x and 1.8x increase in lymph node and spleen lacZ$^+$ cells respectively) as compared to the oil control. In addition, we were able not only to detect recombination/expression by flow cytometry, but also by analysis of lacZ stained tissues sections. As shown in **Figure 19**, we found a number of lacZ positive cells in

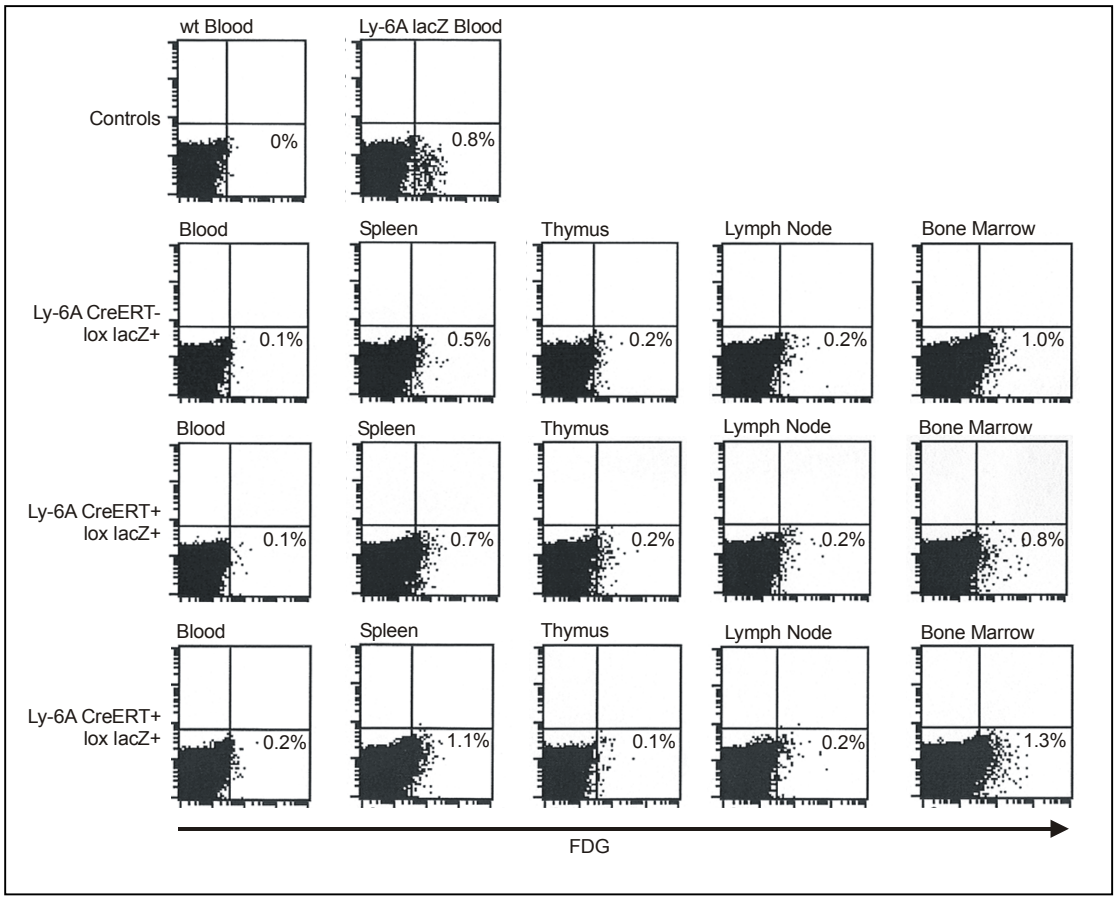

Figure 20. Flow cytometric analysis of hematopoietic tissues from adults acutely treated with Tamoxifen. Adult Rosa26 lox lacZ and Ly-6A CreERT: Rosa26 lox lacZ transgenic mice were treated for 2 weeks with daily injections of 1.4 mg of Tamoxifen.
Two weeks subsequent to the last injection, blood, spleen, thymus, lymph node and bone marrow cells were obtained and stained with FDG. Percentages of cells in the lower right quadrant are indicated. Adult wild type and Ly-6A lacZ blood serves as negative and positive staining controls, respectively.

lymph node and spleen sections, verifying the flow cytometric results. We also found some low level lacZ expressing cells in the bone marrow. However, this may represent background staining, as we observed background staining of bone marrow in this flow cytometric analysis.

As the recombination/expression efficiency of Ly-6A CreERT appeared to be very low, we performed an acute treatment of adult mice with Tamoxifen. Göthert et al. (2005) showed that injection of 2mg Tamoxifen/day for 2 weeks resulted in 11.4 % YFP positive cells in total bone marrow. Thus, we injected double transgenic Ly-6A CreERT: Rosa26 lox lacZ and Ly-6A CreERT: Rosa26 lox EGFP adult mice and appropriate controls with acute doses of Tamoxifen. In the experiment with acute Tamoxifen treatment of double transgenic Ly-6A CreERT: Rosa26 lox lacZ mice (n=2), we injected only 1.4 mg of Tamoxifen/day for 14 days because of the young age (7 weeks old) of the mice. As a negative control, a single transgenic adult (Rosa26 lox lacZ) was treated identically with Tamoxifen. Two weeks following the last injection (to allow for hematopoietic recovery), FDG-flow cytometric analysis was performed on the bone marrow, spleen, thymus, lymph nodes and peripheral blood of all three mice (**Fig. 20**). Slight increases in the percentages of FDG positive cells were found in double transgenic peripheral blood, spleen and bone marrow. The spleen showed the highest percentages of FDG positive cells: 0.7% and 1.1% in double transgenics as compared to 0.5% in the single transgenic control. Thus, some recombination/expression occurred.

We also treated Ly-6A CreERT: Rosa26 lox EGFP adult mice (n=2) with a total of 28 mg of Tamoxifen (2mg/day for 14 days). As a negative control, a single transgenic adult (Rosa26 lox EGFP) was identically treated with Tamoxifen. Two weeks following the last injection, flow cytometric analysis for GFP expression was carried out on several hematopoietic organs (**Fig. 21**). However, we did not observe any significant increase in the percentage of GFP expressing cells even after such acute Tamoxifen treatment. Thus, as found in our *in utero* treatment studies, Tamoxifen treatment of Ly-6A CreERT: Rosa26 lox lacZ adults results in some low level detectable recombination/expression of the lacZ reporter. However, no detectable reporter recombination/expression occurs in Ly-6A CreERT: Rosa26 lox EGFP Tamoxifen-treated mice.

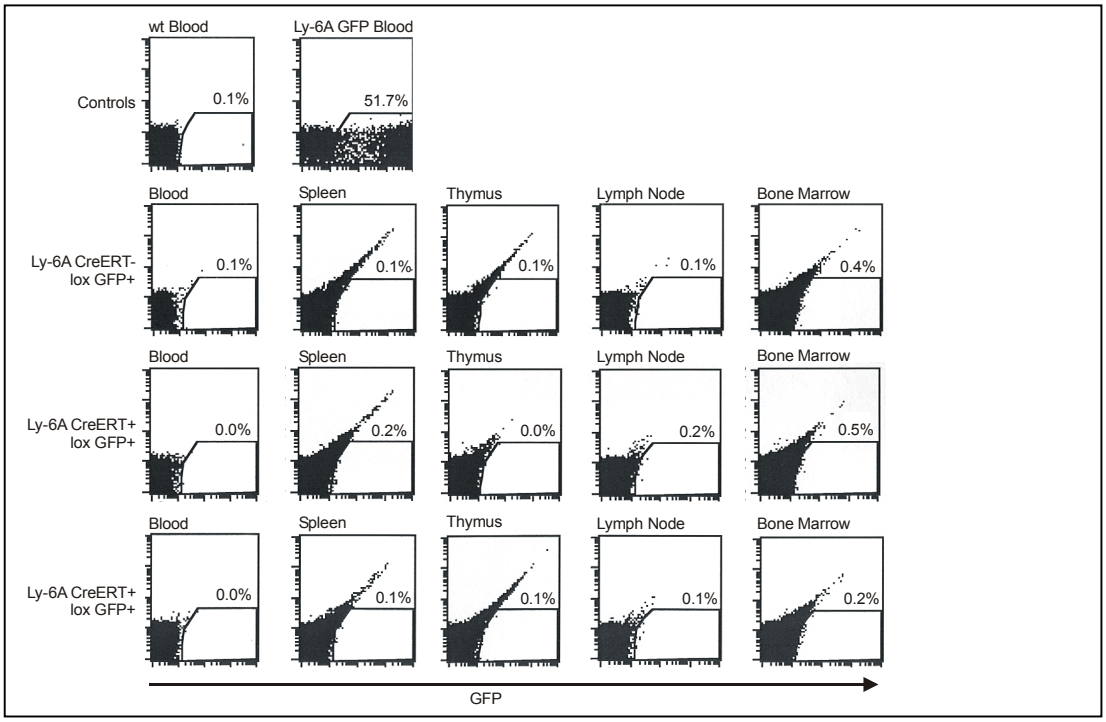

Figure 21. Flow cytometric analysis of hematopoietic tissues from adults acutely treated with Tamoxifen. Adult Rosa26 lox EGFP and Ly-6A CreERT: Rosa26 lox EGFP transgenic mice were treated for 2 weeks with daily injections of 2 mg of Tamoxifen.
Two weeks subsequent to the last injection, blood, spleen, thymus, lymph node and bone marrow cells were obtained and GFP fluorescence was measured by flow cytometry. Percentages of cells in the lower right quadrant are indicated. Wild type and Ly-6A GFP blood served as negative and positive staining controls, respectively.

DISCUSSION

Despite expression of Cre in appropriate embryonic and adult hematopoietic tissues at appropriate times, only rare recombination/expression events were observed. The rare reporter expressing cells were found in the expected hematopoietic tissues: AGM, yolk sac and bone marrow. However, due to background in FL and YS, it was difficult to determine whether these tissues contained any recombined cells. Thus, we were unable to answer our question with certainty concerning the migration of HSCs from the AGM and the colonization of fetal liver and the adult hematopoietic tissues with these cells. More efficient recombination/expression is needed for these data to be reliable. It is uncertain whether the problem lies in the efficiency of recombination (level of Cre expression, activity of Cre) or the level of expression of the recombination reporter gene or both.

Efficiency of obtaining embryos/adults after Tamoxifen treatment

Due to reports that Tamoxifen dose affects the viability of embryos and health of dams in maintaining pregnancy, we examined our results to determine the efficiency of obtaining embryos after administration of various doses and dosage regimens of Tamoxifen. As summarized in **Table 1**, we found that we were able to harvest 50% to 100% viable E13 and E14 embryos after a single i.p. injection of 2-4 mg of Tamoxifen into pregnant dams. This efficiency decreased to 20% when 6 mg of Tamoxifen was administered at E10. Moreover, 2 injections of 3-4 mg of Tamoxifen per day did not appear to affect embryo viability or the health of the pregnant dam. Hence, the administration of greater quantities of Tamoxifen is best performed in multiple injections rather than a single high dose administration.

Table 1. Effect of i.p. Tamoxifen treatment on pregnancy and viability of midgestation embryos.

Day of injection	Day of analysis	Tamoxifen dose	Number of injected females	Number of females with offspring	Number of healthy offspring/total	% healthy offspring
E9	E13	2 mg	2	2	9/10	90%
		3 mg	2	2	21/21	100%
		4 mg	4	1	3/6	50%
		6 mg	1	0	NA	NA
E10	E13	4 mg	3	1	5/5	100%
		6 mg	2	1	1/5	20%
E10+E11	E14	3 mg (x2)	1	1	6/6	100%
		4 mg (x2)	3	3	24/30	68%
Total			18	11	69/83	83%

We also summarized our data concerning the number of full term pregnancies and number of healthy offspring obtained after Tamoxifen administration (**Tab. 2**). The administration of high Tamoxifen dose (8mg) appears to affect maintenance of the pregnancy. Comparisons of E9 injections with E10 injections show similar rates of pregnancy maintenance, but a decreased percentage of healthy offspring from the E10 injections. Only one out of 4 offspring from a pregnant dam injected 3 times with Tamoxifen was healthy. Thus, while it is difficult to determine the precise nature of these variations, a simple explanation may be difficulties in the injection procedure as pregnancy proceeds; i.e. injection directly into the embryo instead of the peritoneal cavity of the dam. Also, since E9 and E10 injections were performed at the initial stages of our experimentation, these injections may not have been performed with the same accuracy as the double and triple injections that were performed most recently.

When the consequences of Tamoxifen injection on the maintenance of pregnancy were examined, we found that 59% of pregnancies were maintained until E13/14 (11/18), while 56% (35/68) were maintained until E18. Injection of oil (the oil (the vehicle for administration of Tamoxifen) resulted in 75% of females maintaining their pregnancy until E18.

Table 2. Effect of i.p. Tamoxifen treatment on pregnancy and viability of offspring at term.

Day of injection	Day of analysis	Tamoxifen dose	Number of injected females	Number of females with offspring	Number of healthy offspring /total	% healthy offspring
E9	E18	0 mg	4	2	15/19	79%
		3 mg	9	5	9/13	69%
		4mg	4	3	19/20	95%
		8mg	1	0	NA	NA
E10	E18	0 mg	4	4	11/24	49%
		4 mg	11	6	17/35	49%
E9+E10	E18	3 mg (x2)	3	3	11/18	61%
		1 mg (x2)4OHT	3	3	1/22	5%
E10+E11	E18	3 mg (x2)	1	1	0/2	0%
		4 mg (x2)	23	13	10/48	21%
		1 mg (x2)4OHT	1	0	NA	NA
E13+E14	E18	4 mg (x2)	6	3	10/22	46%
E9+E10+E11	E18	4 mg (x3)	4	1	1/4	25%
Total		Control (oil)	8	6	25/43	58%
		Tamoxifen	62	35	78/160	49%
		4OHT	4	3	1/22	5%

We often found vaginal bleeding in late gestation and also at the time of delivery. Thus, Tamoxifen injection does affect the health of the pregnant dam. Surgical delivery greatly increased out ability to obtain viable offspring, and this procedure and our fostering program greatly improved with experience. Hence, most of our data in **Table 2** reflect the results from surgical delivery of offspring.

We observed that the viability of offspring obtained after *in utero* treatment is better at midgestation than at term. 58% of offspring obtained from control oil injected pregnant dams were healthy at term. However, we obtained only 49% healthy offspring at term when Tamoxifen was injected. Considering that we obtained 83% viable midgestation embryos after Tamoxifen treatment of pregnant dams, it appears that the injection process as well as the injection of Tamoxifen decreases the percentage of viable offspring obtained at term.

Finally, we compared the effects of i.p. administration of Tamoxifen with that of 4OHT (a Tamoxifen derivative). We found that 4OHT is quite toxic. Only 1 out of 22 (5%) offspring survived at term after 4OHT treatment as compared to a 21-61 % survival rate of offspring after Tamoxifen injection. Taken together, these data suggest that Tamoxifen treatment does affect the maintenance of pregnancy and the viability of offspring. Despite treatment difficulties, our optimized midgestation injection procedure (high dose Tamoxifen administered on 2-3 consecutive days) yielded sufficient numbers of mice to perform lineage tracing studies.

Efficiency of Tamoxifen induced recombination

Regardless of the dose and different Tamoxifen treatment protocols (including acute injections), we were able to detect at various developmental stages only rare recombination marker-expressing cells in hematopoietic tissues using the Rosa26 lox lacZ reporter line. The low number of recombined/lacZ expressing cells is most likely explained by a very inefficient Ly-6A CreERT-mediated recombination. In turn, this very low efficiency in genetic marking of hematopoietic cells may result from either insufficient expression levels or reduced recombination activity of CreERT fusion protein, thus leading to few recombination events *per se* (Vooijs et al., 2001). Theoretically, to perform recombination, only 4 Cre molecules are needed (Branda and Dymecki, 2004). A possible low or mosaic expression of Tamoxifen-inducible CreERT recombinase then may affect the number of cells able to recombine loxP elements. Mosaic expression of transgenes has been shown to be related to the integration site of the transgene (Ly-6A CreERT) in the genome, namely position effect variegation (McMorrow et al., 2000; Weber et al., 2001). The recombination activity of CreERT might be affected by possible structural changes (point mutations affecting active site in structure of Cre or ligand binding component) (Weber et al., 2001) or inappropriate protein folding, masking the active or binding sites.

In addition, as suggested from other studies using inducible Cre/loxP system, differences *in vivo* in local concentrations of inducible ligands such as Tamoxifen or 4OHT may also affect the level of Cre-mediated recombination (Hayashi and McMahon, 2002). Tamoxifen or 4OHT were administrated by intraperitoneal injection of the pregnant dam and thus different hematopoietic tissues most likely receive different amounts of these drugs through the circulation. Interestingly, while it is estimated that 4OHT binding to the LBD of the estrogen receptor occurs a 1 to 100-fold higher binding affinity than Tamoxifen, it is very surprising that we did not find a high frequency in the recombination/expression frequency of the marker genes *in vitro* (Williams et al., 1994; Charlier et al., 1995). However while the binding affinity is higher, it has been demonstrated that a single i.p. injection of the same dose (1mg) of 4OHT and Tamoxifen into pregnant females at 9.5 day post coitum gave similar recombination/expression results (Danielian et al., 1998). Notably, our data showed that *in utero* treatments with 4OHT were much more toxic than Tamoxifen (Guo et al., 2002), leading to abortion. In addition to less toxicity, Tamoxifen is more soluble in oil (a carrier) than 4OHT, making it more consistent in prepared concentration. Nonetheless, in the future to increase the efficiency of Ly-6A CreERT-directed recombination additional structural improvements of this fusion protein need to be considered. Also, we need to test more Ly-6A Cre transgenic lines to eliminate the chances of position effect variegation in expression of Cre.

Variation in the expression of reporters after Cre-loxP recombination

Initially in our studies, we used a dual reporter transgenic line, Z/EG (Novak et al., 2000), which should allow for the marking of both unrecombined (lacZ positive) and recombined cells (GFP positive). Unfortunately, *in vivo* fate mapping experiments with this marker transgenic line failed. While we could detect some lacZ positive (unrecombined) hematopoietic cells at different developmental stages, we did not detect any GFP positive in sections of embryonic hematopoietic tissues, nor in more sensitive flow cytometry analysis of double transgenic (Ly-6A CreERT: Z/EG) hematopoietic tissues isolated from neonates (n=2) treated with Tamoxifen *in utero*. Furthermore no GFP expressing cells were detected by flow cytometry in peripheral blood from *in utero* Tamoxifen-treated adult double transgenic mice (Ly-6A CreERT: Z/EG). Since it was known that the Dhh Cre transgene effectively induces recombination in many tissues in Rosa26 lox lacZ compound transgenic mice, we used these transgenic mice to test for recombination and expression of the Z/EG GFP marker. This reporter was found not to be expressed in the expected lineages. Thus, we continued out experiments using the Rosa26 lox lacZ and Rosa26 lox EGFP lines.

Table 3. Comparative analysis of lacZ expression in different adult hematopoietic tissues by flow cytometry.

genotype	peripheral blood (PB)	thymus	lymph nodes (LN)	spleen (SP)	bone marrow (BM)
wt	0%	0%	0%	0.1%	0.3%
Rosa26 lox lacZ	0%	0%	0.1%	0.2%	0.2%
CAGcre:Rosa26 lox lacZ	24%	78%	38%	51%	47%
Ly-6A lacZ	0.5%	1%	4%	3%	2%

The fact that expression of these recombination markers were not highly expressed in our studies prompted us to test whether we should expect high levels of reporter expression per cell. To test the levels of reporter expression, we analyzed different hematopoietic organs from double transgenic adult mice carrying the CAG Cre transgene and the Rosa26 lox lacZ or EGFP reporters. The CAG promoter is ubiquitously and constitutively active beginning at the 2-4 cell stage of embryogenesis. In such compound transgenic mice, lacZ expression was detectable by FDG-FACS.

As shown in **Table 3** high percentages of lacZ expressing cells were found in all hematopoietic tissues. Background was very low in Rosa26 lox lacZ single transgenic mice. As a positive control, Ly-6A lacZ mice were also analyzed. In these mice, fewer cells were FDG positive, as expected. Thus, the Rosa26 lox lacZ marker is recombined and expressed efficiently in the presence of ubiquitously and constitutively expressed Cre, strongly suggesting that lacZ reporter expression levels are not responsible for the low frequency of recombination/expression lacZ positive cells found in our experiments

with compound Ly-6A CreERT: Rosa26 lox lacZ mice. Instead, it is most likely that Cre-mediated recombination was inefficient.

Similarly, we tested for the expression of GFP in CAG Cre: Rosa26 lox EGFP mice. While we found high percentages of bone marrow cells to be GFP positive (59%), the levels of GFP expression per cell were very low as compared to the background observed in Rosa26 lox EGFP single transgenic bone marrow and extremely low as compared to GFP expression levels per cell observed in Ly-6A GFP bone marrow. Thus, the low level of GFP expression per cell in the recombined Rosa26 locus, is most likely responsible for our inability to detect even a few recombined expressing cells that we would expect from our results in Ly-6A CreERT: Rosa26 lox lacZ mice.

Taken together, our data form a starting basis for future fate mapping experiments to determine which tissue(s) during ontogeny lead to the generation of the adult hematopoietic system. We have found lacZ recombination marker expressing cells (that were marked during midgestational stages in the AGM region) at a low but detectable frequency in the adult hematopoietic system. As recombination was inefficient, we will test newly generated Ly-6A CreERT transgenic lines to find the most efficient CreERT expressing line that is not subject to position effect variegation. New CreERT constructs will also be tested in attempts to find a more active fusion protein. In addition, according to Göthert et al., (2005) it appears that the levels of EYFP reporter expression per cell are greater than the levels we found in the Rosa26 lox EGFP mice. Thus, we will use the Rosa26 lox EYFP line in the next round of experiments.

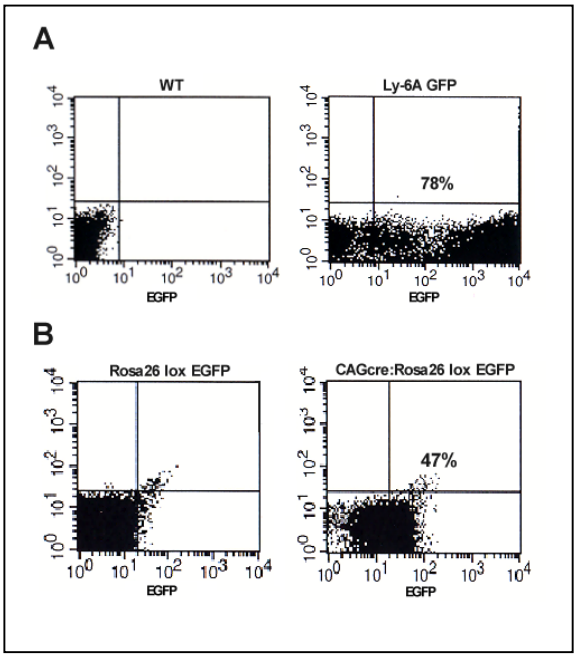

Figure 22. Flow cytometric analysis for expression of recombination marker (GFP) in adult spleen.
Adult spleen cells from double transgenic (CAGcre: Rosa26 lox EGFP) mouse (right panel **B**) or from single transgenic mouse lacking CAGcre transgene as a negative control (left panel **B**) were analyzed for presence of GFP expressing cells. As a positive GFP control, peripheral blood from adult Ly-6A GFP was taken (right panel **A**). For a negative control, peripheral blood from wild type mouse was included (left panel **A**). The percentage of GFP expressing cells is indicated, where x-axis corresponds to GFP fluorescence intensity. In the panel **B** the shift of GFP expressing cells is observed (right dot-plot), although the intensity of EGFP fluorescence is not high.

MATERIALS and METHODS

Generation of embryos

For generating embryos, timed matings between Ly-6A CreERT males and reporter females were set up in the late afternoon. The day of vaginal plug detection was counted as embryonic day 0. Plugged mice were killed by cervical dislocation and embryos were subsequently placed in PBS supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. Embryonic tissues were dissected according to procedure described before (Müller et al., 1994; Dzierzak and de Bruijn, 2000). All animals were housed in agreement with institutional guidelines, with food and water *ad libitum*. Animal procedures were performed in compliance with the Standards for Human Care and Use of Laboratory Animals.

Genotyping of embryos/mice

Genomic DNAs were isolated from the tails/or embryonic tissues of Ly6AcreLBD, Rosa26 lox lacZ (R26R, Soriano, 1999) and Rosa 26 lox EGFP (Mao et al., 2001) lines by phenol/chloroform extraction and isopropanol/NaCl precipitation. For detection of the *Cre* transgene, DNA (~100ng) was subjected to PCR reaction (initial 5 min denaturation step, followed by 30 cycles of denaturation (1min at 94°C), annealing (1min at 55°C) and elongation (2min at 72°C), and final elongation (5 min at 72°C)) using forward primer (5'-ACCCTGTTACGTATAGCCGA-3') and reverse primer (5'- CTCCGGTATTGAAACTCCAG-3') to generate a PCR product size of 416bp. For genotyping of Rosa 26 lox lacZ transgene PCR was done for 30 cycles (94°C, 1min; 55°C, 2 min and 72°C, 2min), to obtain 320bp-product using *lacZ*-specific primers: (CATZ1) 5'-GCGTTACCCAACTTAATCG-3' and (CATZ2) 5'-TGTGAGCGAGTAACAACC-3'. To verify the homozygous/or heterozygous state of these mice, an additional PCR was performed (94°C, 4 min of initial denaturation, followed by 34 cycles of 94°C, 1min; 65°C, 1.2 min and 72°C, 1.2 min; and final elongation step-72°C, 10 min) using a set of three primers: 5'-GCGAAGAGTTTGTCCTCAACC-3', 5'-GGAGCGGGAGAAATGGATATG-3'and 5'-AAAGTCCTGAGTTGTTAT, which give 2 products: wild type (~550bp) and transgenic (~250bp). Detection of an *EGFP* DNA fragment (440bp) was performed by PCR (5 min at 92^{o}C; followed by 30 cycles of 1 min at 92 ^{o}C, 2 min at 55 ^{o}C and 2 min at 72 ^{o}C; additional extension 5 min at 72 ^{o}C), using *EGFP*-specific primers 5'AAACGGCCACAAGTTCAGCG3' and 5'GGCGGATCTTGAAGTTCACC3'. For all the *cre*, *lacZ* and *EGFP* PCR reactions, an internal DNA control PCR for the presence of housekeeping gene, myogenin was included using following primers: forward 5'-TTACTGCCATCGTGGACAGC-3' and reverse 5'-TGGGCTGGGTGTTAGTCTTA-3' to amplify a 250bp-fragment. This PCR was performed using the same conditions as for *EGFP* genotyping.

Recombination PCR analysis

Although not reported in this chapter, we did attempt to detect recombination products in cells from double transgenic animals and tissues treated with Tamoxifen. However, no positive results were found. The procedure was as follows. PCR reaction was performed on DNA isolated from Tamoxifen/or oil treated embryonic or adult tissues. The primers used in PCR reaction were corresponding to Rosa26 5' fragment (R26F2; 5'-AAAGTCGCTCTGAGTTGTTAT-3') and 5'end of β-galactosidase gene (Z3; 5'-GGCCTCTTCGCTATTACG-3') (Rijnkels et al., 2001; Agah et al., 1997). DNA was initially denatured at 94°C for 3 min, followed by 40 cycles of denaturation (94°C for 1 min), annealing (for 2 min at 58°C) and extension (for 3 min at 72°C), and final elongation (7 min at 72°C) (Rijnkels et al., 2001). The size of the expected recombined PCR product was 600 bp. We also performed a nested (2-step) PCR. In the first-step PCR the following primers were used: forward (R26F2; 5'-AAAGTCGCTCTGAGTTGTTAT-3') and reverse (Z2; 5'-TGTGAGCGAGTAACAACC-3') to amplify a 867bp-fragment. 30µl of the total PCR reaction volume (50µl) was used as a template for the step-2 PCR, in which primers (RS3; 5'-CTCGTGATCTGCAACTCCAGTC-3') and (Z3; 5'-GGCCTCTTCGCTATTACG-3') were used in the same conditions as described above.

Explant culture

Embryonic hematopoietic tissues (YS, AGM or AGM with vitelline and umbilical vessels and gut attached, FL, vitelline and umbilical vessels) dissected from E11 double heterozygous embryos (Ly-6A CreERT: Rosa26 lox EGFP or Ly-6A CreERT: Rosa26 lox lacZ) embryos were placed separately onto sterile Durapore membranes filters (0.65µm; DV Millipore), supported with sterile stainless-steel mesh stands in the 6-well plates. To induce Cre-mediated recombination the tissues were cultured for 3 days in myeloid long-term culture medium (M5300; Stem Cell Technologies) containing IL-3 (100ng/ml), 1µM 4OHT and 10^{-5}M hydrocortisone succinate (Sigma) at the air-liquid interface at 37 ^{o}C in 5%CO_2. After 2 days the medium (containing IL-3 and 4OHT) was refreshed. For the negative recombination control the tissues were cultured for 3 days in the medium without 4OHT.

Cell preparations and flow cytometry analysis

Single cell suspensions of isolated embryonic tissues were obtained by incubation with collagenase (0.125%; Sigma, type I) for 1 hour at 37 ^{o}C in PBS containing 10% FCS and penicillin/streptomycin. Cells were dispersed by gentle pipetting, filtered, washed and resuspended again in PBS containing 10% FCS and penicillin/streptomycin (400µl of total volume). For adult hematopoietic organs, single cell suspensions were prepared by mechanic disruption of the tissues and subsequent filtering trough cell strainer (40µm, BD Falcon), washing and resuspension of the cells in PBS supplemented with 10% FCS and penicillin/streptomycin. Just before flow cytometric measurement, 7AAD (7-aminoactinomycin D, Molecular Probes) was added

to cell suspensions (containing at least $1x10^6$ cells) to discriminate between alive and dead cells. Flow cytometry analysis was performed on a FACScan (BD Biosciences) using Cell Quest software for data analysis. Range of accumulated events was between $5x10^4 – 3x10^5$.

Transplantations

After explant culture, donor AGMs (Ly-6A CreERT: Rosa26 lox EGFP) were collagenase treated, filtered, washed and resuspended in sterile PBS. Cells were injected intravenously into wild type male recipients (C57BL/10 x CBA)F1. Before transplantation these recipients were irradiated with a split dose (3h interval) of 900 rad in total from a ^{137}Cs source. For short-term survival of the recipients, $2x10^5$ spleen cells from adult (C57BL/10 x CBA)F1 mice were co-injected. Transplanted mice were kept in cages with filter-top and received drinking water supplemented with neomycin (1.6g/l) for at least 1 month. For analysis of donor-cell engraftment, peripheral blood was taken from the recipients after 1month (short-term) and 4months (long-term) and donor marker (EGFP) specific PCR was performed as described previously (de Bruijn and Dzierzak, 2002).

RT-PCR

Total RNA was prepared from embryonic or adult hematopoietic tissues using TRIZOL (Gibco/ Life Technologies) according to the manufacturer instructions. To remove DNA from RNA preparations the samples were treated with RQ1 RNase free DNAse (Promega) and finally RNA was dissolved in DEPC-water. Concentration of RNA was estimated by OD measurement (ND-1000 Spectrophotometer, NanoDrop Technologies). 1μg of tRNA was used in RT reaction with Superscript II reverse transcriptase (Invitrogen/Life Technologies) for cDNA synthesis according to the protocol supplied by the manufacturer. To test for *Cre* expression, RT-PCR was performed using *Cre*-specific primers already described in the genotyping section with the same PCR conditions. For semi-quantitative analysis (normalization control), an additional *β-actin* RT-PCR was done (92°C, 5 min of initial denaturation, followed by 30 cycles of 92°C, 45s; 58°C, 45s and 72°C, 1.3 min; and final elongation step-72°C, 7 min) using these primers: forward 5'-ATGGATGATGATATCGCCGC-3' and reverse 5'-GCGCTCGGTGAGGATCTT-3'. All the RT-PCR reactions were run on agarose gels with ethidium bromide (EtBr), scanned with the Typhoon (Molecular Dynamics) and analyzed using ImageQuant software.

X-Gal / BluO-Gal staining

Murine embryos or tissues (E11-E18) or adult hematopoietic organs were washed once with washing solution (PBS/0.02% NP-40) to remove serum. Next the tissues or embryos were fixed at 4°C from 2 to 24hrs (depending on the size/stage) in fixative containing 1% formaldehyde, 0.2% gluteraldehyde, 2mM $MgCl_2$, 5mM EGTA and 0.02% NP-40. After fixation, the tissues, embryos or organs were washed three

times for 30 minutes in washing solution at room temperature. Staining was performed using 2 different substrates: X-Gal or BluO-Gal depending on the embedding procedure: X-Gal for Tissue-Tec frozen embedded and BluO-Gal for paraffin embedded tissues or embryos. Staining was carried out in the dark overnight at 37°C or at room temperature in staining solution containing 5mM $K_3Fe(CN)_6$, 5mM $K_3Fe(CN)_6 \times 3H_2O$, 2mM $MgCl_2$, 0.01% Nadeoxycholate, 0.02% NP-40 with X-Gal or BluO-Gal (1mg/ml). The next day tissues or embryos were washed a few times (5x) in PBS and subsequently fixed in 4% formaldehyde at 4°C for overnight. Afterwards they were embedded in paraffin or fast frozen in Tissue-Tek (O.C.T. Sakura Finetek), sectioned (10-12μm) and counterstained with hematoxylin. For mounting the sections of mouse embryos or tissues Entallan (Merck) /or Pertex (HistoLab) were used.

Fluorescent microscopy analysis

Embryos or hematopoietic tissues (AGM, liver, yolk sac, bone marrow, spleen, thymus or lymph nodes) were fixed in 4% PFA /PBS (para-formaldehyde in PBS) at 4°C from 2 – 24 hrs depending on the developmental stage. Next they were equilibrated in 20% sucrose solution (in PBS) overnight at 4°C, fast frozen in Tissue-Tek and sectioned (10-12μm) using a microtome (Jung CM 3000 cryostat, Leica). Air-dried slides were washed 2 times in PBS for 5 min. at room temperature, dehydrated in series of ethanol (50 – 100%), and finally mounted using Vectashield medium (Vector Laboratories, Burlingame, CA).

FDG staining

To detect β-galactosidase (lacZ+) positive cells, single cell suspensions of hematopoietic (embryonic or adult) tissues were prepared and $1x10^6 – 2x10^6$ of the cells in 50μl of DMEM medium supplemented with 10% FCS and penicillin/streptomycin were stained with FDG (fluorescein di-β-D-galactopyranoside; Molecular Probes). To count the cells a Bürker hemocytometer was used. To exclude dead cells Trypan blue (Sigma) was applied to the cells. Before loading the FDG, the cells suspensions were prewarmed in a 37°C water bath for 5 min. Next the 50μl of FDG was added to the cell suspension (50μl) and reaction was allowed to proceed for exactly 1 min. After 1 min. the uptake of FDG was stopped by adding 700μl of ice-cold DMEM medium containing 10% FCS and penicillin/streptomycin and placing the tubes with the cells on ice. Hydrolysis reaction was allowed to continue for 1 hour in the dark at room temperature. Before flow cytometric measurement, 7AAD (see above) was added to the cell suspensions to exclude the dead cells. Prior to FDG staining, peripheral blood (PB) and embryonic placenta were first fractionated on a ficoll (Lymphoprep, Axis-Shield, Norway) gradient to eliminate a large number of erythroid cells.

Preparation and administration of Tamoxifen or 4OHT (4-hydroxy-Tamoxifen)

Tamoxifen and 4OHT (Sigma) were prepared as stock solutions at the concentrations 40mg/ml and 10mg/ml respectively. The stock solutions were prepared

by first addition of the Tamoxifen or 4OHT to 100% ethanol, followed by addition of sterile sunflower oil (1 ethanol: 9 oil volume ratio). After short manual mixing and prewarming (5min in 37°C water bath), the Tamoxifen or 4OHT suspensions were placed in an ultra-sonicator water bath (Soniprep 150, Sanyo). The stock solutions were kept at -20°C for months (4OHT) and 6 months (Tamoxifen). Tamoxifen or 4OHT were injected into the pregnant females or adult mice intraperitonally (75-100μl). In the case of 4OHT injections the solutions were sonicated just before the use to avoid precipitation. Sterile sunflower oil was injected as a negative control. For *in vitro* experiments, only 4OHT was used. The stock solution of 4OHT (1mM) was prepared by dissolving the 4OHT in 100% ethanol. The concentration of 4OHT used in the bone marrow cell cultures or explant cultures of hematopoietic embryonic tissues was 1 or 5μM.

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CHAPTER 3

Multipotential hematopoietic progenitor cells from embryos developed *in vitro* engraft unconditioned W^{41}/W^{41} neonatal mice

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****equal contribution to this work***

Chapter 3

Multipotential hematopoietic progenitor cells from embryos developed *in vitro* engraft unconditioned W^{41}/W^{41} neonatal mice

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Background and Objectives. The first hematopoietic stem cells (HSC) in the mouse able to give rise to the adult hematopoietic system emerge at embryonic day (E) 10.5 in the intraembryonic aorta-gonads-mesonephros (AGM) region, as demonstrated by transplantation into irradiated adult recipients. It has been shown by transplantation into conditioned neonatal or hematopoietic mutant adult recipients that less potent multipotential hematopoietic progenitors exist in the mouse embryo at E9, one day earlier than the appearance of HSC. Studies of the lineage relationships of multipotential hematopoietic progenitors and HSC in the mouse embryo have been complicated by inaccessibility due to *in utero* development. Attempts are being made to create an *in vitro* whole mouse embryo culture system to access the developing mouse embryo for such studies of hematopoietic cell emergence during early and mid-gestational stages. The aim of this study was to compare the development of multipotential hematopoietic progenitors in early *in utero* and *in vitro*-developed mouse embryos.

Design and Methods. To test hematopoietic progentior/stem cell activity in the mouse embryonic tissues obtained from genetically marked *in utero* and *in vitro*-developed embryos, transplantations were performed using unconditioned neonatal W^{41}/W^{41} (c-kit hematopoietic mutant) recipients. Long-term donor-cell reconstitution in transplanted mice was measured by (i) semiquantitative polymerase chain reaction and (ii) flow cytometry on peripheral blood and hematopoietic organs.

Results. Our experimental data show that multipotent hematopoietic progenitors from *in utero*-developed embryos engraft unconditioned W^{41}/W^{41} neonates. Furthermore, *in vitro*-developed whole embryos also contain early multipotent hematopoietic progenitor cells that are able to yield high-level, long-term engraftment of W^{41}/W^{41} neonates.

Interpretations and Conclusions. The *in vitro* culture of whole mouse embryos during mid-gestational stages allows for the normal growth of multipotential hematopoietic progenitors that can be assayed by transplantation into W^{41}/W^{41} neonatal recipients. Thus, *in vitro*-developed whole embryos can be used with confidence in future studies to examine the lineage relationships of multipotential hematopoietic progenitors and HSC.

Chapter 3

Key words: hematopoietic stem cells • W^{41}/W^{41} • neonates • development • AGM •embryo culture

Introduction

Over the last ten years, hematopoietic stem cell (HSC) development has been studied using several *in vivo* transplantation models. The first generation of HSCs with engraftment potential equivalent to adult bone marrow (BM) HSC has been shown in the aorta-gonad-mesonephros (AGM) region at embryonic day (E) 10.5 using irradiated adult recipients (Medvinsky and Dzierzak 1996, Muller*, et al* 1994). Prior to E10.5, no adult repopulating HSCs were detected. However, multipotential hematopoietic progenitors have been detected in earlier stage yolk sac (E9 YS) and para-aortic splanchnopleura (E9 Sp) when tested in busulfan-conditioned neonatal recipients by intravenous injection or injection into the hematopoietically active neonatal liver (Yoder and Hiatt 1997, Yoder*, et al* 1997a, Yoder*, et al* 1997b). These cells are thought to represent an early type of HSC that, through further inductive interactions, will acquire adult BM homing and/or high proliferative properties. Similarly, multilineage or single lineage repopulating cells were observed when pre-E10.5 YS or Sp cells were injected into Rag-/-:γc-/- and W locus (receptor tyrosine kinase c-kit) lymphopoietic/hematopoietic mutant (adult or embryonic stage) recipients (Abramson*, et al* 1977, Cumano*, et al* 2001, Fleischman*, et al* 1982, Fleischman and Mintz 1979, Fleischman and Mintz 1984, Toles*, et al* 1989, Weissman*, et al* 1977). It is now of great interest to further study and manipulate multipotential hematopoietic progenitors in mouse embryos developed *ex utero*. *In vitro* culture conditions for whole mouse embryos have been established and allow for overt normal development over a 24-48 hour period (Cockroft, 1990). However, to date no studies have been performed to determine whether hematopoietic development is normal in embryos cultured for more than 12 hours (Sugiyama*, et al* 2003). The W^{41}/W^{41} mouse strain offers advantages as a transplant recipient for further studies on embryonic onset of multipotential hematopoietic progenitor cell activity. Previously, it has been shown that neonates with severe W mutations (in the absence of irradiation) can be effectively engrafted with donor fetal liver HSCs (Capel and Mintz, 1989). However, while the most severe mutations of the W locus affect both hematopoiesis and fertility (Geissler*, et al* 1981, Nocka*, et al* 1990), the mild W^{41} mutation (a single amino acid mutation in the c-kit kinase domain) affects only hematopoiesis (Harrison and Astle, 1991). Thus, it is possible to cross W^{41}/W^{41} homozygous mutant parents to obtain complete litters of homozygous neonates for transplantation assays.

The objective of this study was to examine and compare multipotent hematopoietic progenitor activity in pre-E10.5 embryos developed *in utero* and *in vitro*,

in order to determine wheter *in vitro* whole embryo cultures allow the normal growth of these rare progenitors. We used unconditioned W^{41}/W^{41} newborn mice as transplant recipients. The resulting hematopoietic reconstitution of neonatal W^{41}/W^{41} recipients with cells from embryonic tissues was tested at 2 to 12 months post-transplantation by polymerase chain reaction (PCR) for donor cell genetic markers and by flow cytometric analysis.

Design and Methods

Mice and embryo generation

W^{41}/W^{41} neonates were obtained from W^{41}/W^{41} mating pairs. C57BL/6 or (129Sv x C57BL/6)F1 adult mice (2-4 months old) from our facility were used as recipients for secondary transplantation experiments. Timed matings of C57BL/6 females with actin-EGFP male transgenic mice (Okabe*, et al* 1997) were performed for the generation of the embryos. The day of vaginal plug discovery was considered embryonic day 0 (E0). Embryos were genotyped by EGFP fluorescence. This transgenic line was maintained on a C57BL/6 background. All mice were housed in the Erasmus Medical Center Animal unit according to the institution guidelines, with food and water provided *ad libitum*. Pregnant dams were sacrificed by cervical dislocation at E8 until E10 and embryos were isolated from the uterus as described previously (Dzierzak and de Bruijn, 2002). Embryos were staged by counting the number of somite pairs.

Embryo culture

Whole embryos were cultured *in vitro* as previously described (Cockroft, 1990; New*, et al* 1973). Briefly, E8, E8.5 or E9 embryos were dissected so as to remove the mural trophoblast and Reichert's membrane while maintaining the integrity of the extra-embryonic membranes (amnion and yolk sac with the ectoplacental cone). Pools of 2-5 E8 or E8.5 embryos or 1-4 E9 embryos were cultured in a glass roller flask. E8/E8.5 embryos were cultured in Hank's buffered salt solution (HBBS) containing 50% rat serum, and E9 embryos in 100% rat serum with glucose levels adjusted to 10mM. Rat serum came from adult male and female rats and was prepared immediately by centrifugation after blood collection. Only those lots that gave maximal embryo growth were used. Oxygenation of the cultures varied between 20% O_2 (E8/E8.5) and 40% O_2 (E9). Cultures were maintained in a 38^{o}C incubator for 24 hours. Embryos were harvested from the cultures and assessed for growth and viability under a dissection microscope. Only those embryos demonstrating overtly normal physiology and blood circulation were further processed. Dissection of tissues and preparation of cell suspensions for injection was performed as described previously (Dzierzak and de Bruijn, 2002).

Transplantation

Cell suspensions (20µl in phosphate-buffered saline) were injected into livers of W^{41}/W^{41} neonates (within 24 hours after birth) with a pulled glass pipette (Narishige Scientific Instrument Ltd, GD-1; 1 x 90 mm). At 2, 6 and 12 months post-transplantation, donor cell contributions were analyzed by semi-quantitative polymerase chain reaction (PCR) and flow cytometry for the EGFP transgene. In some cases, BM cells obtained from positive engrafted primary recipients were transplanted into secondary recipients (129Sv x C57BL/6)F1 or C57BL/6 irradiated (9 Gy) (Dzierzak and de Bruijn, 2002).

Briefly, genomic DNA was isolated from peripheral blood of transplanted mice: 100ng of DNA was used for PCR using EGFP primers 5'AAACGGCCACAAGTTCAGCG3' and 5'GGCGGATCTTGAAGTTCACC3' and myogenin primers 5'TTACGTCCATCGTGGACAGC3' and 5'TGGGCTGGTGTTAGTCTTA3'. DNA was subjected to an initial 5 minute denaturation at 92^{o}C followed by 30 cycles of denaturation (1 min at 92 ^{o}C), annealing (2 min at 55 ^{o}C) and elongation (2 min at 72 ^{o}C). Reactions were run on an ethidium bromide stained agarose gel. EGFP and myogenin fragment sizes are 440 bp and 250 bp respectively. The percentage donor-cell contribution was determined by a dilution series of transgenic and normal genomic DNA from which a standard curve was established.

Ethidium-stained fragments were analysed by ImageQuant using the myogenin fragment as the DNA normalization control.

Flow cytometric analysis

Flow cytometric analysis was performed to detect hematopoietic cells in the embryos developed *in utero* and *in vitro* and also to detect the presence and lineage of EGFP-positive cells in recipient mice, as described previously (de Bruijn*, et al* 2002; Dzierzak and de Bruijn, 2002). Phycoerythrin-conjugated B220 (CD45R, RA3-6B2), CD4 (GK1.5), CD8 (53-6.7), Mac1 (CD11b, M1/70), TER119 and Gr1 (8C5) specific antibodies (Pharmingen) were used to detect mature lineage hematopoietic cells. Single cell suspensions were stained with antibodies for 30 minutes on ice, washed twice in phosphate-buffered saline with 10% fetal calf serum, and filtered through a nylon mesh prior to screening. 7AAD was used to exclude dead cells during analysis on a FACScan (Becton-Dickinson). Ten thousend events were analyzed.

RESULTS

Multipotential hematopoietic progenitors engraft W^{41}/W^{41} newborn recipients

Sp/AGM and YS tissues were dissected from E9, E9.5 and E10 EGFP transgenic embryos, prepared as single cell suspensions and injected into unconditioned W^{41}/W^{41} neonates. At 2, 6 and 12 months post-transplantation, donor cell engraftment

was measured by PCR (EGFP transgene) or flow cytometric analysis on peripheral blood samples. As shown in **Table 1**, engraftment (12 months post-transplantation) was observed in the peripheral blood of 4 out of a total of 34 W^{41}/W^{41} recipients injected. Low-level engraftment from E9 Sp, E10 AGM or E10 YS cells (approximately 1%) was found in three recipients. Interestingly, high-level engraftment was observed as early as 2 months and up to 12 months post-transplantation in one recipient receiving E9.5 Sp cells (3 embryo equivalents; ee).

By flow cytometric analysis we found high-level multilineage donor contribution in the circulation of this recipient at 12 months post-transplantation (61% of $B220^+$, 43% of $CD4^+/8^+$, 53% of $Mac1^+$, 34% of $TER119^+$, 27% of $Gr1^+$ were donor-derived). This recipient was then sacrificed and hematopoietic tissues were examined. **Figure 1A** shows high-level engraftment ranging from 13% to 78% in BM, thymus, lymph node and spleen in the granulocyte ($Gr\text{-}1^+$), macrophage ($Mac\text{-}1^+$), T lymphoid ($CD4/8^+$; may include dendritic cells) and B lymphoid ($B220^+$) lineages. To test for the self-renewal of donor-derived HSC, BM cells (5, 1.3 and 0.6 x 10^6 cells) were transplanted into irradiated secondary adult recipients. FACS of peripheral blood more than 4 months post-transplantation showed high-level engraftment in all recipients (**Table 1**). This engraftment was multilineage as determined by DNA PCR and flow cytometric analysis on cells obtained from hematopoietic tissues (*not shown*). Thus, as measured by transplantation into W^{41}/W^{41} neonates, the E9.5 Sp region of *in utero* developed embryos contains a small number of potent long-term multipotential hematopoietic cells with self-renewal abilities.

In vitro-cultured whole embryos contain multipotential hematopoietic progenitors

Given the inaccessibility of mammalian embryos for *in utero* manipulation, it is of great interest to culture whole mouse embryos *in vitro* to follow their development. After a culture period of 24-48 hours, embryos are typically scored for viability and normal development by several anatomical landmarks-neural tube closure, somite pairs, circulating blood, etc. To determine whether hematopoietic development is maintained in *in vitro*-cultured mouse embryos, we performed flow cytometric analysis. E9 embryos were obtained and used for flow cytometric analysis or cultured overnight to the E10 stage and then analyzed. Sp/AGM and YS tissues were dissected and cells stained with antibodies specific for hematopoietic markers: CD45 for all hematopoietic cells, Mac-1 for macrophages, Ter119 for erythrocytes (the most common circulating hematopoietic cells at this stage) and Sca-1 for mainly hematopoietic stem cells. As shown in **Table 2**, there are relatively abundant levels of hematopoietic cells in both the AGM and the yolk sac of cultured embryos. While there are slight decreases in the percentages of some hematopoietic cells in the Sp/AGM region of the embryo proper,

yolk sac percentages of some hematopoietic cells are slightly increased during the culture period. Thus, despite the substantial growth of the embryo body during the 24-hour culture period, the hematopoietic cell content of the AGM and YS is generally maintained.

Table 1. *Presence of multipotential hematopoietic progenitors in embryos developed in utero or in an in vitro embryo culture.*

	Somite pairs	ee injected	Number repopulated/Number transplanted (% donor cell engraftment)					
			Sp/AGM			Yolk Sac		
			2 mo	6 mo	12 mo	2 mo	6 mo	12 mo
In utero								
E9.0	17-21	0.5-1	1/7 (1)	1/7 (8)	1/7 (1)	0/6	0/6	0/6
E9.5	22-26	2-3	1/2 (50)	1/2 (50)	1*/2 (50 [a])	0/2	0/2	0/2
E10.0	30-34	1-2	0/11	0/10	1/10 (1)	0/7	0/6	1/7 (1)
***In vitro* whole embryo culture**								
E8 to 9	15-22	1-2	0/9	0/9	0/9	0/8	0/8	0/8
E8.5 to 9.5	25-29	2	0/2	0/2	1/2 (1)	0/2	0/2	0/2
E9 to 10	26-34	1	1/13 (10)	ND	1*/12 (27 [b])	3/11 (80,10,1)	ND	2*/9 (85 [c],10)

Secondary transplantation*	Number repopulated/Number transplanted (% donor cell engraftment)			
# of injected cells	5×10^6	3×10^6	1.3×10^6	0.6×10^6
E9.5 AGM [a]	3/3 (92-100)	-	2/2 (45-100)	2/2 (30-72)
E9 to E10 AGM [b]	-	2/3 (3, 7)	-	-
E9 to E10 YS [c]	-	3/3 (15, 21, 16)	-	-

Embryos were obtained directly from pregnant females (*in utero*) or cultured (*in vitro*) for 24 hours. More than 80% of *in vitro*-cultured whole embryos were viable and met the criteria for obvious normal development. Only these embryos were used in the transplantation experiments. Tissues were dissected and single cell suspensions injected into the liver of unconditioned newborn W^{41}/W^{41} recipients. Peripheral blood DNA was obtained at 2, 6 and 12 months post-transplantation and tested by semiquantitative PCR for the presence of the EGFP transgene. Secondary transplantations were performed with varying doses of bone marrow cells isolated from the recipients indicated by the asterisks [a, b, c]. Cells from [a] 3 pooled AGMs (22-26 sp), [b] 1 AGM (33 sp) and [c] 1 YS (32 sp) were injected into primary neonatal recipients. Some tertiary repopulation was also observed (not shown). Sp = somite pairs; mo=months; ee = embryo equivalents.

To test more specifically whether hematopoietic development in *in vitro* cultured mouse embryos is comparable to that of *in utero*-developed embryos, we tested for the presence of multipotential hematopoietic progenitors by transplantation into unconditioned W^{41}/W^{41} recipients. E8, E8.5 and E9 EGFP transgenic whole embryos were cultured for 24 hours. Only viable, anatomically normal embryos that developed to the E9, E9.5 and E10 stages (respectively) were used for transplantation. Sp/AGM, and

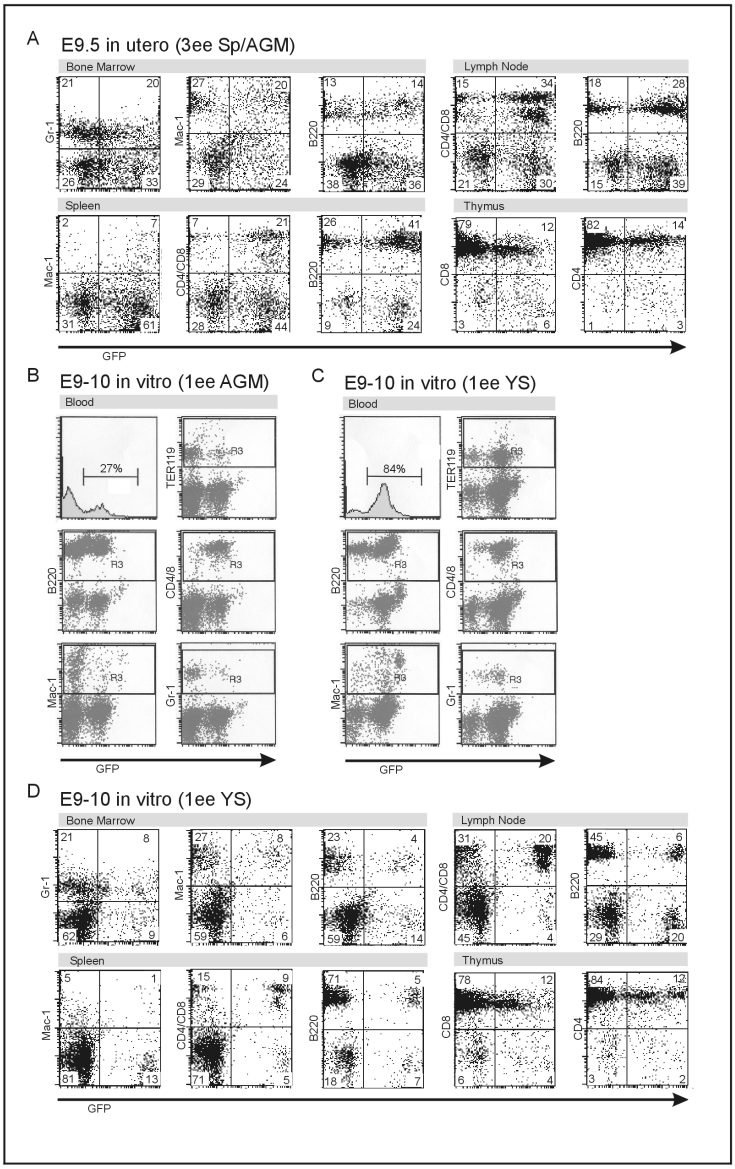

Figure 1. Flow cytometric analysis showing high-level, multilineage repopulation of unconditioned newborn W^{41}/W^{41} recipients more than 12 months post-transplantation. BM, thymus, lymph node, spleen and/or blood cells were incubated with fluorescent antibodies specific for granulocytes (Gr-1), macrophages (Mac-1), T lymphocytes (CD4 and CD8), B lymphocytes (B220) and erythroid lineage cells (TER119) and fluorescence profiles measured on a FACScan (Becton-Dickinson). A. Tissue engraftment of recipient injected with Sp/AGM cells from pooled *in utero* developed embryos (E9.5, 22-26 sp, 3 ee injected). B. Peripheral blood engraftment of a recipient injected with AGM cells from a whole embryo *in vitro*-cultured from E9 to E10 (33 sp, 1 ee injected). C. Peripheral blood engraftment and D. Tissue engraftment of a recipient injected with YS cells from a whole embryo *in vitro* cultured from E9 to E10 (32 sp, 1 ee injected). Percentages of donor ($EGFP^+$) and recipient ($EGFP^-$) lineage specific cells within the total viable population are indicated in the upper quadrants. Sp = somite pairs; ee = embryo equivalents.

YS tissues were dissected and cell suspensions were injected into unconditioned W^{41}/W^{41} neonates. Three out of a total of 42 injected recipients were found to contain at least 10% donor-derived cells in the peripheral blood at 12 months post-transplantation (**Table 1**). One recipient was engrafted with E10 AGM cells and two recipients were engrafted with E10 YS cells that were obtained from E9 embryos cultured for 24 hours (26 to 34 somite pairs; sp). Flow cytometric analysis revealed multilineage hematopoietic engraftment in the peripheral blood (**Figure 1B** and **1C**) and the hematopoietic tissues of these recipients (**Figure 1D**). Furthermore, transplantation of

primary BM into irradiated adult secondary recipients yielded long-term multilineage repopulation (**Table 1**). Hence, we conclude that AGM and YS multipotential hematopoietic progenitors develop under *in vitro* whole embryo culture conditions and that these cells are self-renewing.

Table 2. *Percentages of cells positive for hematopoietic cell surface marker expression before and after whole embryo culture (E9 to E10).*

	Sp/AGM		YS	
	% before culture (E9)	% after culture (E10)	% before culture (E9)	% after culture (E10)
CD45	3.2 (1.6-5.0)	1.9 (1.2-2.5)	10.4 (9.4-11.7)	20.7 (20.0-21.3)
Mac-1	1.6 (1.2-2.3)	0.5	6.6 (6.3-6.9)	10
Ter119	2.4	1.6	9.1	12
Sca-1	1.5 (1.4-1.5)	0.6 (0.3-0.8)	2.1 (1.0-3.0)	2.6 (0.6-4.5)

Percentages are the average of 1-4 flow cytometric analyses (range).

Discussion

These results represent two major advances in the study of developmentally early, multipotential, self-renewing hematopoietic cells. First and most importantly, the 24-hour *in vitro* whole embryo culture system allows for the development of these immature hematopoietic stem cells. When the 12-month engraftment results are considered, the numbers of multipotential hematopoietic progenitors in the *in vitro* cultured embryos appear to be equivalent (or slightly increased) to those in the *in utero* developed embryos. Cells from the *in utero*-developed embryos engraft 3% (1/34) of the injected W^{41}/W^{41} neonatal recipients to high levels (greater than or equal to 10% donor derived hematopoietic cells) while cells from *in vitro*-cultured embryos were able to engraft 7% (3/42) of W^{41}/W^{41} neonatal recipients to these high levels. We show that both *in utero* and *in vitro* developed progenitors are capable of long-term repopulation, producing all hematopoietic lineages. Furthermore, these cells lead to repopulation of secondary (and even tertiary, *not shown*) recipients, confirming that they possess self-renewal capacity, an important property of stem cells. Thus, our results demonstrate for the first time that the 24-hour *in vitro* whole embryo culture system effectively allows for the development and/or maintenance of these complex hematopoietic progenitors and that this system can be used for further progenitor manipulation and fate mapping studies.

While we would have used the *in vitro* culture system for the study of the development of adult repopulating HSC within the AGM and YS (and the subsequent

colonization of the fetal liver by these cells) our studies are limited here to neonatal repopulating multipotential hematopoietic progenitor cells. Disappointingly, the *in vitro* whole embryo culture system does not promote the normal development of embryos past the 34 to 35 somite pair stage (mid E10), the time during which the first adult repopulating HSCs are generated. It is currently thought that during midgestation the placenta takes over important functions leading to further embryo growth and perhaps even the *de novo* generation of adult type HSC (Gekas, *et al* 2005; Ottersbach and Dzierzak, 2005; Dzierzak and de Bruijn, 2002). Thus, until the *in vitro* whole embryo culture system is further improved, fate mapping is restricted to only the early multipotential hematopoietic progenitors.

The second advance of these studies is the demonstration that unconditioned W^{41}/W^{41} neonates can be used (although not as efficiently as busulfan-conditioned neonates) as transplantation recipients for the detection of multilineage repopulating cells from early stage embryos. Whether from *in utero* and *in vitro* developed embryos, the high levels of repopulation we found in W^{41}/W^{41} recipients contrast with those resulting from transplantation of early AGM cells into Rag-/-:γc-/- adult recipients (Cumano*, et al* 2001). In Rag-/-:γc-/- adult recipients, pre-E10.5 AGM cells (2.5-3 ee of explant cultured Sp [0-10 sp] or AGM [30-35 sp] yielded only low levels (1-5%) of granulocytic repopulation. In the more comprehensively studied busulfan-conditioned neonate transplantation model (Yoder*, et al* 1997a), repopulation with E9 Sp and YS was much higher (ranging from 8-27%), was multilineage, and was more comparable to the levels we observed in W^{41}/W^{41} neonatal recipients. The frequency of repopulated W^{41}/W^{41} neonatal recipients we observed was much lower than reported for busulfan conditioned neonatal recipients (Yoder*, et al* 1997a). Although the reason for this is not yet known, it may be due to less endogenous hematopoietic cell competition in conditioned neonates versus the W^{41}/W^{41} neonates. Busulfan conditioning is thought to reduce the number of endogenous hematopoietic progenitors to negligible levels. Future comprehensive studies are necessary to determine the actual level of the hematopoietic progenitor content of W^{41}/W^{41} embryos. The results of such studies may reveal that mild conditioning of W^{41}/W^{41} neonates will yield a higher frequency of repopulation.

In addition, progenitor cell enrichments (de Bruijn*, et al* 2002, Sanchez*, et al* 1996) by flow cytometric sorting may more efficiently reveal multipotential hematopoietic cells when injected into W^{41}/W^{41} neonatal recipients. Notwithstanding the efficiency of the W^{41}/W^{41} neonatal transplantation model, the transplanation results reported here, together with the demonstration that *in vitro*-cultured embryos contain multipotential hematopoietic progenitors, represent advances in the study of the development of the adult hematopoietic system. The next major challenge is to generate a functional fate map by marking the presumptive hematopoietic precursor cells in

whole embryo cultures with low density lipoproteins labelled with a fluorescent dye (LDL-DiI).

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CHAPTER 4

GATA-2 Plays Two Functionally Distinct Roles during the Ontogeny of Hematopoietic Stem Cells

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Chapter 4

GATA-2 Plays Two Functionally Distinct Roles during the Ontogeny of Hematopoietic Stem Cells

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Abstract

GATA-2 is an essential transcription factor in the hematopoietic system that is expressed in hematopoietic stem cells (HSCs) and progenitors. Complete deficiency of GATA-2 in the mouse leads to severe anemia and embryonic lethality. The role of GATA-2 and dosage effects of this transcription factor in HSC development within the embryo and adult are largely unexplored. Here we examined the effects of *GATA-2* gene dosage on the generation and expansion of HSCs in several hematopoietic sites throughout mouse development. We show that a haploid dose of *GATA-2* severely reduces production and expansion of HSCs specifically in the aorta-gonad-mesonephros region (which autonomously generates the first HSCs), whereas quantitative reduction of HSCs is minimal or unchanged in yolk sac, fetal liver, and adult bone marrow. However, HSCs in all these ontogenically distinct anatomical sites are qualitatively defective in serial or competitive transplantation assays. Also, cytotoxic drug-induced regeneration studies show a clear *GATA-2* dose–related proliferation defect in adult bone marrow. Thus, GATA-2 plays at least two functionally distinct roles during ontogeny of HSCs: the production and expansion of HSCs in the aorta-gonad-mesonephros and the proliferation of HSCs in the adult bone marrow.

Key words: GATA-2 • hematopoietic stem cells • AGM • haploinsufficiency • gene dosage

Abbreviations used in this paper: AGM, aorta-gonads-mesonephros; CFU-GM, colony-forming unit–granulocyte macrophage; CFU-S11, colony-forming unit–spleen; E, embryonic day; ER, estrogen receptor; ES, embryonic stem; FL, fetal liver; 5-FU, 5-fluorouracil; GFP, green fluorescent protein; HSC, hematopoietic stem cell; LSK, $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$; YS, yolk sac.

Introduction

Hematopoietic stem cells (HSCs) at the foundation of the adult hematopoietic differentiation hierarchy have the ability to self-renew and produce all the distinct blood

cell lineages (1, 2). HSCs capable of complete long-term hematopoietic repopulation of irradiated adult recipients are first generated in the aorta-gonads-mesonephros (AGM) region at mid-embryonic day (E) 10.5 (3, 4) and localize to the dorsal aorta, vitelline, and umbilical arteries (5). Subsequently, at mid/late E11, HSC activity is also found in the yolk sac (YS) and fetal liver (FL). HSC activity increases significantly in E11 AGM and E12 YS when they are cultured as whole tissue explants for 3 d (3, 6). Although the spatial and temporal appearance of HSCs during development has been described and quantitated, the molecular mechanisms underlying HSC generation, expansion, and maintenance are not well explored. One molecule important during hematopoietic ontogeny is GATA-2, a member of the GATA family of zinc finger transcription factors (7, 8). RT-PCR analysis shows high expression of *GATA-2* in adult hematopoietic progenitor cells and HSCs (9, 10). Furthermore, immunohistochemistry, *in situ* hybridization, and transgenic analyses show *GATA-2* expression as early as E8 in the para-aortic splanchnopleura (precursor tissue to AGM) and subsequently in the AGM (11, 12). In the E11.5 AGM, GATA-2 is expressed in the aortic endothelium and neighboring mesenchymal cells, which are both considered putative hemogenic cell populations. Embryos lacking GATA-2 are anemic, have moderately reduced numbers of primitive erythroid cells and hematopoietic progenitor cells (13), and die at E10.5, the time of HSC induction and expansion. Due to this lethality, the role of GATA-2 has been examined mainly by *in vitro* colony-forming assays of cells from early embryonic tissues and hematopoietic cultures of *GATA-2*$^{-/-}$ embryonic stem (ES) cells. In both cases, hematopoietic progenitor numbers are severely reduced. Further studies in chimeric mice produced with *GATA-2*$^{-/-}$ ES cells show no contribution of the mutant cells to any hematopoietic tissues. Together, these data indicate that GATA-2 is crucial for the maintenance, proliferation, and/or survival of immature hematopoietic progenitors (13).

The function of GATA-2 had also been studied through enforced overexpression achieved by retroviral transduction or transfection of genes encoding either a wild-type GATA-2 or an inducible GATA-2–estrogen receptor (ER) fusion protein (12, 14–19). Irrespective of these attempts, a simple conclusion on the function of GATA-2 in the HSCs or progenitor cells is difficult. On one hand, expression of the inducible form of GATA-2–ER fusion protein in the multipotent hematopoietic progenitor cell line FDCP promotes differentiation (17). On the other hand, constitutive expression of GATA-2 in murine BM cells blocked progenitor-derived colony formation (14). The opposing results may be due to the cell types chosen for these experiments. However, it is also suggested that the biochemical behavior of the artificially generated GATA-2–ER fusion protein may not be the same as the wild-type unmodified GATA-2 protein (18). Thus, the most relevant data on *GATA-2* dose effects on hematopoiesis may be best obtained within the physiological context of the whole organism wherein *GATA-2* is

expressed under the endogenous regulatory machinery in the appropriate cell types.

The study of transcription factor dose and function at the earliest stages of hematopoietic development is of particular interest for an understanding of HSC generation. Studies on the *runx1* transcription factor have shown that a haploid dose results in changes in HSC induction, expansion, and distribution in the midgestation mouse embryo (20). Moreover, haploinsufficiency of human Runx1 (AML-1) results in thromobocytopenia and a propensity to develop myeloid leukemia (21). Recently, a correlation between a reduction in *GATA-2* expression and aplastic anemia (22) has been demonstrated. Hence, to further understand the role of GATA-2 in the ontogeny of HSCs we examined the effects of *GATA-2* haploinsufficiency on induction and expansion of HSCs during development by *in vivo* hematopoietic transplantation assays and phenotypic analysis of compound transgenic embryos (*GATA-2*$^{+/-}$*–Ly-6A GFP*) (23).

Here we present data showing that the numbers of hematopoietic progenitors in *GATA-2*$^{+/-}$ embryos are reduced. More importantly, we observe a dramatic quantitative reduction in HSC activity specifically in *GATA-2*$^{+/-}$ AGMs and a further reduction in the serial repopulating ability of these HSCs. In contrast, *GATA-2*$^{+/-}$ HSC numbers appear quantitatively normal in the adult BM but are qualitatively defective in the setting of competitive transplantation. In addition, *GATA-2*$^{+/-}$ HSCs exhibit a delay in regeneration of the hematopoietic system after cytotoxic drug challenge, suggesting that GATA-2 levels play a role in HSC proliferation. Thus, GATA-2 plays functionally distinct roles in the production of HSCs in the AGM region and the proliferation of HSCs throughout ontogeny.

Materials and Methods

GATA-2 Mutant Mice and Embryos. *GATA-2* mutant mice (13) were backcrossed onto the C57BL/6 background for over 10 generations and were housed in the Erasmus Medical Center Animal unit according to the institution guidelines with food and water provided ad libitum. The day of vaginal plug discovery from overnight matings (GATA-2$^{+/-}$ male X C57BL/6 GATA-2$^{+/+}$ or GATA-2$^{+/-}$ female) was counted as day 0. Pregnant dams were killed and embryos isolated from the uterus as described previously (24). Embryos (E10–E11) were staged by counting somite pairs (25). Genotyping was performed by PCR as described previously (13). Compound transgenic embryos were obtained by mating Ly-6A GFP hemizygous (23) and GATA-2$^{+/-}$ mice.

Dissection, Explant Culture, Cell Preparation, and In Vivo Trans-plantation. Dissections, tissue explants, and cell preparation were performed as described previously (24). Recipient mice (C57BL/6 or [129Sv X C57BL/6] F1 females, 8–16 wk old) received a split dose of 1,000 rad (for colony-forming unit–spleen [CFU-S_{11}]), 900 rad (for HSCs), or 640 rad (for

competitive repopulation assay) at a 3-h interval from a ^{137}Cs source on the day of donor cell injection. Cells were injected i.v. into the tail veins. Except for CFU-S, serial, and competitive transplantation assays, 2 X10^5 female spleen cells from the recipient strain were coinjected to provide short-term survival. Secondary transplantations were performed with 3 X 10^6 BM cells from the primary recipients. Cell dose for competitive repopulation assays was 3 X 10^5–3 X 10^7. Injected animals were provided with 0.16% Neomycin (Sigma-Aldrich)-supplemented water. For CFU-S_{11}, recipients were killed at 11 d posttransplantation by cervical dislocation, spleens isolated, and microscopic colonies scored after fixing with Teleynesnicki's solution overnight.

Semiquantitative PCR for Donor Contribution. Blood, tissue, or specific cell lineage DNA (100 ng) was used for semiquantitative PCR to detect the donor HSC contribution to the recipient. For male-derived donor cells, *YMT*-specific PCR (350-bp product) was used together with myogenin-specific PCR (250 bp) for DNA normalization. The detection of *GATA-2* mutant–derived donor cells was performed with *GATA-2/NEO* (950 bp) and GATA-2 wild-type (600 bp) PCR. Primers and PCR conditions were as previously described (4, 13).

Cell Sorting and Flow Cytometry Analysis. FACS was performed on a FACS Vantage SE (Becton Dickinson) (23), and flow cytometric analyses were performed on a FACSCalibur dual laser instrument (Becton Dickinson) with CellQuest software (BD Bioscience). Staining of embryonic tissue cell suspensions was performed in PBS supplemented with 10% FCS, and 2 μg/ml 7AAD (Molecular Probes) was added for dead cell exclusion. Staining of adult HSCs was performed in PBS supplemented with 0.5% BSA. Biotin-conjugated anti–Gr-1 (ER-MP20) was a gift from Dr. P.J.M. Leenen (Erasmus MC, Rotterdam, The Netherlands). All other antibodies were obtained from BD Biosciences including APC-conjugated anti–c-kit (clone 2B8), PE-conjugated anti–Sca-1 (clone D7), PerCP-Cy5.5–conjugated anti-CD8a (clone 53–6.7), anti-B220 (clone RA3-6B2), anti-CD19 (1D3), anti-CD11b (anti–Mac-1, clone M1/70), and biotin-conjugated anti-CD3 (145-2C11), anti-CD4 (H129.19), Ly-76 (TER-119), anti-IgM (II/41), and anti-NK1.1 (PK136). A secondary step was sometimes performed with streptavidin-conjugated PerCP-Cy5.5 (BD Biosciences).

Immunohistochemistry. Embryos were fixed for 30 min with 2% paraformaldehyde/PBS at 4ºC and equilibrated in 20% sucrose/PBS overnight at 4ºC. They were immersed in Tissue Tek, quick frozen on dry ice, and stored at -80ºC until ready for use. Serial cryosections (10 μm) were treated in 100% cold acetone for 10 min, washed three times with PBS (0.05% Tween), blocked with 0.5% BSA and 50% vol/vol Avidin D block solution (Vector Laboratories) for 15 min, washed three times, blocked with Biotin blocking solution (Vector Laboratories) for 15 min, and washed three times. Subsequently, sections were incubated with a biotin-conjugated anti-CD34 (clone RAM34; BD Biosciences) diluted 1:50 in 1% BSA/0.05% Tween/PBS at room temperature for 1 h, washed three times, incubated with the detection reagent Streptavidin-Cy5 (Rockland) diluted 1:500 in 1% BSA/0.05% Tween/PBS at room temperature for 30 min, washed three times, dehydrated in ethanol (from 70 to 100%), and mounted with vectashield (Vector Laboratories). Confocal images were taken of every tenth section starting caudally at the point where the urogenital ridges first appeared up to the rostral bifurcation of the dorsal aorta.

5-Fluorouracil Treatment. *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ mice (9–10 wk old) were i.v. injected with 150 mg 5-fluorouracil (5-FU; Sigma-Aldrich) per 10 g body weight. Treated mice were then killed at 4, 8, 12, and 16 d posttreatment, and BM cells were isolated and analyzed by flow cytometry and *in vitro* culture assay.

Progenitor Colony Assay. BM cells were plated in triplicates from 2 X 10^4 to 5 X 10^5 cells per plate in methylcellulose medium (Methocult GF M3434; StemCell Technologies Inc.) supplemented with stem cell factor (SCF), IL-3, IL-6, and Epo. All cultures were incubated at 37ºC in a humidified chamber under 5% CO2. Colony-forming unit–granulocyte macrophage (CFU-GM) were scored with an inverted microscope at day 7 of the culture.

Results

GATA-2*$^{+/-}$ *AGM and YS Explants Contain Fewer CFU-S$_{11}$. To investigate if *GATA-2* gene dosage affects the production of hematopoietic progenitors during development, colony-forming unit spleen activity was assayed at 11 d postinjection (CFU-S_{11}) so as to measure the more immature erythro-myeloid progenitors. AGM, YS, and FL were isolated from *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ embryos at E10.5–E12, explant cultured, and cells transplanted into irradiated adult recipients (**Fig. 1**). At all time points tested, CFU-S_{11} activity was detected both in *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ AGM, YS, and FL explants (**Fig. 2**). As expected from previous data (3), high numbers of E10.5 CFU-S_{11} are first detected in the *GATA-2*$^{+/+}$ AGM explants, they increase at E11, and thereafter decline in number. In *GATA-2*$^{+/-}$ AGM and YS explants, CFU-S_{11} were reduced by three- to ninefold and one- to fourfold, respectively, compared with wild-type tissues. In contrast, CFU-S_{11} activity in *GATA-2*$^{+/-}$ FL explants was normal. No reductions in FL CFU-S_{11} numbers were observed at any time. Therefore, *GATA-2* gene dosage affects the generation and/or proliferation of immature hematopoietic progenitor cells in the YS and AGM of the midgestation embryo.

HSC Activity Is Severely Reduced in the GATA-2*$^{+/-}$ *AGMs. To investigate if *GATA-2* dose also affects midgestation HSC development, we performed the most stringent functional HSC test: the long-term, high level, multilineage repopulation of irradiated adult mouse recipients. E11 and E12 *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ AGM, YS, and FL cells were transplanted directly into irradiated adult recipients (**Fig. 1**). Engraftment by *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ cells was assayed by semiquantitative PCR of the male Y chromosome–specific marker *Ymt* and the *GATA-2/NEO* mutant allele (respectively) in recipient peripheral blood DNA at 4 mo posttransplantation. Only those recipients showing >10% engraftment with donor-marked cells were considered positive for high level repopulation. PCR results of one representative experiment are shown in **Fig. 3**. Briefly, each recipient received one-third of the cells obtained from an individually prepared E12 tissue (0.33 tissue equivalents). At 4 mo postinjection, progeny of transplanted *GATA-2*$^{+/+}$ AGM cells were found in the peripheral blood of five out of

eight recipients (*Ymt* PCR). In contrast, reduced HSC activity was found in E12 HSC in *GATA-2*$^{+/-}$ AGMs. Only one out of six recipients was highly engrafted (*GATA-2* mutant and *Ymt* PCR). Similarly, recipients transplanted with YS cells revealed some reduction of HSC activity in *GATA-2*$^{+/-}$ embryos. However, no reduction in HSC acitivity was found in *GATA-2*$^{+/-}$ FLs. Further analysis of recipients repopulated with *GATA-2*$^{+/-}$ cells revealed high level multilineage engraftment within all hematopoietic tissues (blood, thymus, LNs, BM, and spleen) and subsets tested (splenic T and B lymphocytes, erythroid and myeloid cells) (not depicted).

The cumulative results of all transplantation experiments are shown in **Table I** and reveal that at both E11 and E12, HSC activity is severely reduced in *GATA-2*$^{+/-}$ AGMs. The percentage of recipients repopulated with E11 *GATA-2*$^{+/-}$ AGM cells is

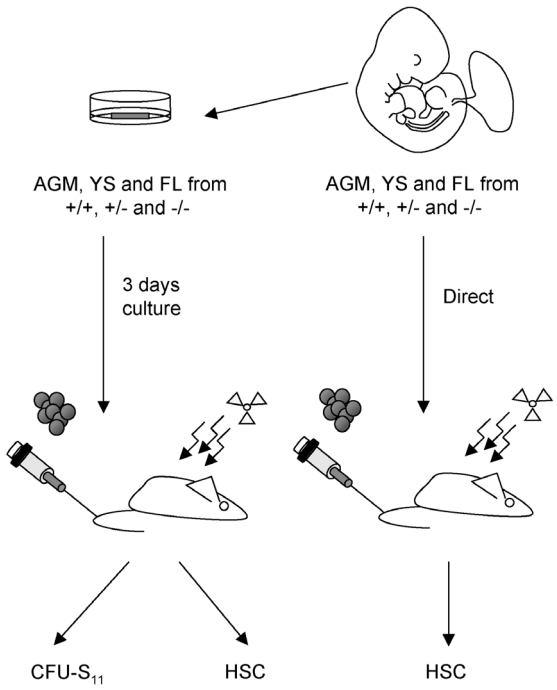

Figure 1. Strategy for studying HSCs and hematopoietic progenitor cells in *GATA-2* mutant embryonic tissues. *GATA-2*$^{+/+}$,*GATA-2*$^{+/-}$,and *GATA-2*$^{-/-}$ AGM regions, YS, and FL were harvested from midgestation mouse embryos. In some cases (left) they were then cultured for 3 d as whole tissue explants before preparation of single cell suspensions and injection into irradiated adult recipients to assay for CFU-S_{11} or HSCs. In some cases (right), single cell suspensions were prepared directly from freshly isolated tissues and injected into irradiated adult recipients for HSC activity.

only 6%, whereas 25% of recipients are repopulated with E11 *GATA-2*$^{+/+}$ AGM cells. This represents a greater than fourfold decrease in HSC activity in E11 *GATA-2*$^{+/-}$ AGMs. Furthermore, at E12 *GATA-2*$^{+/-}$ AGMs are ninefold reduced in HSC activity compared with *GATA-2*$^{+/+}$ AGMs. Reductions in the HSC activity of *GATA-2*$^{+/-}$ YS and FL tissues are less severe and stage dependent. The percentage of mice repopulated by E11 *GATA-2*$^{+/-}$ YS (23%) is comparable to that of *GATA-2*$^{+/+}$ YS (28%), and the FL at this stage contains only limited HSC activity. However, at E12 slight reductions in HSC

activity are observed for both *GATA-2*$^{+/-}$ YS and FL (1.6- and 1.3-fold, respectively). Thus, two copies of the *GATA-2* gene are required for the normal generation, expansion, and/or survival of HSCs in the AGM region.

Ex Vivo Expansion and Maintenance of AGM HSC Activity Is Sensitive to GATA-2 Dose. Since it was shown previously that HSC activity generated in the AGM can be amplified (either by induction or proliferation) when whole tissues are cultured for 3 d (3), we examined the effects of *GATA-2* gene dosage on HSCs in such explant cultures

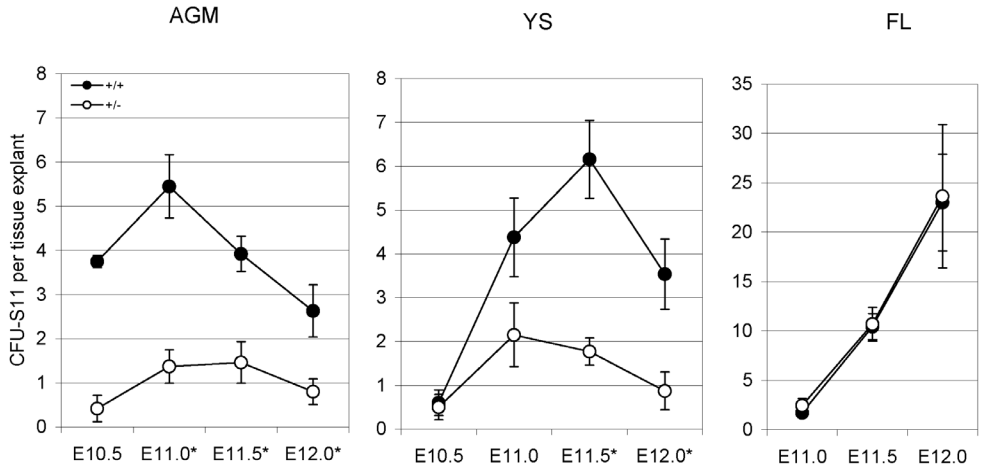

Figure 2. CFU-S_{11} activity in *GATA-2* mutant tissue explants. CFU-S_{11} were assayed from AGM, YS, and FL explants (E10.5–E12.0) after 3 d of culture. Each point represents the average CFU-S_{11} number per embryo tissue equivalent ±SEM detected in the corresponding tissue and genotype. 3–14 independent experiments were performed with 0.2–4.5 tissue equivalents injected per recipient. +/+, *GATA-2*$^{+/+}$; +/-, *GATA-2*$^{+/-}$. The total number of injected AGM explants: E10.5 +/+ = 6, E11.0 +/+ = 21, E11.5 +/+ = 14, E12.0 +/+ = 12, E10.5 +/- = 7, E11 +/- = 22, E11.5 +/- = 12, E12.0 +/- = 12; YS explants E10.5+/+ = 8, E11.0 +/+ = 8, E11.5 +/+ = 11, E12.0 +/ + =10.75, E10.5 +/- = 8, E11.0 +/- = 11.5, E11.5 +/- = 15.5, E12.0 +/- = 9.5; FL explants E11.0 +/+ = 17, E11.5 +/+ =18, E12.0 +/+ = 8.7, E11.0 +/- = 23, E11.5 +/- = 19.5, E12.0 +/- = 8. Embryonic tissues from E10.5 ranged from 36 to 40 somite pairs (sp), E11.0 ranged from 41–47 sp, E11.5 contain >48 sp and E12 contained >60 sp. *Significant difference in the CFU-S_{11} number between *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ tissue explants: AGM E11.0, P< 0.001; E11.5, P< 0.01; E12.0, P< 0.05; YS E11.5, P <0.001; E12, P<0.05. Note that fewer CFU-S_{11} are detected in both the *GATA-2*$^{+/-}$ AGM and YS explants in comparison to the *GATA-2*$^{+/+}$ explants, whereas the FL CFU-S_{11} numbers are unaffected.

of AGM, YS, and FL from *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ embryos. Tissues (E10.5–E12) were dissected, cultured as explants for 3 d, made into a single cell suspension, and injected into irradiated adult recipients (**Fig.1**). Repopulation was measured at 4 mo posttransplantation, and only those recipients showing >10% donor cell multilineage hematopoietic repopulation (measured in several hematopoietic tissues and lineages)

were considered positive. The results are summarized in **Table II**.

Compared with the results of the direct transplantation experiments (**Table I**), *GATA-2*$^{+/-}$ AGM explants were even more severely reduced in the HSC activity. At E10.5, only 8% of recipients receiving *GATA-2*$^{+/-}$ AGM cells were repopulated, representing an eightfold decrease in HSC activity from *GATA-2*$^{+/+}$ AGM cells. The *GATA-2*$^{+/-}$ cell contribution to the various hematopoietic organs (thymus, spleen, LN, and BM) and purified cell lineages (B and T lymphocytes, myeloid and erythroid) was tested and found to be multipotent, thus demonstrating that *GATA-2*$^{+/-}$ AGMs do generate functional HSCs, albeit at much reduced levels. At later developmental time points (E11, E11.5, and E12), HSC activity, although increasing in *GATA-2*$^{+/+}$ AGM explants, is completely absent from *GATA-2*$^{+/-}$ AGM explants. As seen in the *GATA-2*$^{+/+}$ AGM explants, HSC generation and expansion occurs from E10.5 to E11.5, with HSC numbers maintained thereafter (E12). Thus, the severely reduced HSC activity in *GATA-2*$^{+/-}$ AGM explants can be attributed to reduced HSC expansion, survival, and/or homing in the irradiated recipient.

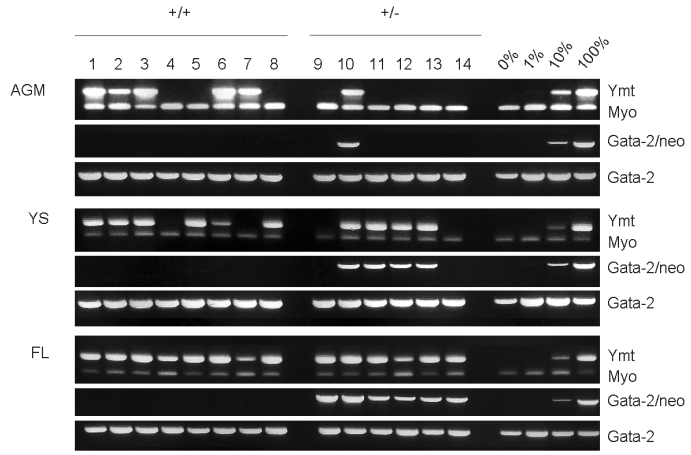

Figure 3. Detection of donor hematopoietic cell contribution in transplantation recipients by peripheral blood DNA PCR analysis. A representative PCR analysis for donor cell contribution to the peripheral blood of transplantation recipients. DNA was isolated from the corresponding recipients (at >4 mo posttransplantation) of *GATA-2*$^{+/+}$, *GATA-2*$^{+/-}$, and *GATA-2*$^{-/-}$ AGM, YS, and FL. Lanes 1–8 and 9–14 are blood DNA samples isolated from recipients receiving cells from E12 *GATA-2*$^{+/+}$ and $^{+/-}$ tissues, respectively. Each sample was analyzed with primers specific for Y chromosome (*ymt*), and *GATA-2* (*GATA-2/NEO* for targeted allele). DNA samples were normalized by PCR with two endogenous gene controls (*myo*, *myogenin*; *GATA-2*, wild-type allele). Control DNA: 0, 1, 10, and 100% represents percentage of the male *GATA-2*$^{+/-}$ DNA mixed with female DNA. Only when the donor marker-specific PCR product was >10%, compared with controls, was the recipient considered to be positive.

The HSC activity of *GATA-2*$^{+/-}$ YS and FL explants was also reduced in

comparison to the *GATA-2*$^{+/+}$ explants. However, this reduction was only slight compared with the AGM. At the first appearance of HSCs in the YS on E11, *GATA-2*$^{+/-}$ YS HSC activity begins to decrease and by E12 is decreased by 1.8-fold from *GATA-2*$^{+/+}$ YS. The decrease in YS explant HSC activity at E12 corresponds with that seen in the directly transplanted YS, suggesting that the expansion but not the maintenance of YS HSC activity is sensitive to *GATA-2* dose.

Similarly, we observed slight reductions in the HSC activity of *GATA-2*$^{+/-}$ FL explants. At the first appearance of HSCs in FL explants on E11.5, HSC activity is reduced from 24% of recipients repopulated with *GATA-2*$^{+/+}$ cells to 11% repopulated with *GATA-2*$^{+/-}$ cells, representing a 2.2-fold decrease in HSC activity. At E12, *GATA-2*$^{+/-}$ FL explants show a 1.7-fold decrease in HSC activity. The changes in FL HSC activity with time are most likely related to the numbers of incoming HSCs from AGM and YS. Thus, these findings suggest *GATA-2* dose affects the expansion but not the survival of HSCs in the FL.

Table I. ***HSC Activity in GATA-2 Mutant Embryonic Tissues.***

	AGM		YS		FL	
stage	+/+	+/-	+/+	+/-	+/+	+/-
E11.0	1[a]/4[b] (25)[c]	1/16 (6)	2/7 (28)	4/17 (23)	0/6 (0)	1/19 (5)
E12.0	11/16 (69)	1/13 (8)	11/15 (73)	6/13 (46)	19/19 (100)	11/14 (78)
2^{o}E12.0	6/6 (100)	0/3 (0)	6/6 (100)	4/9 (44)	6/6 (100)	4/9 (44)

E11 and E12 AGM, YS, and FL tissues were made into a single cell suspension and injected into irradiated adult recipients. Each result represents [a]the number of recipient mice showing donor cells in peripheral blood (DNA) at >4 mo posttransplantation, [b]the total number of mice transplanted, and [c]the percentage of repopulated recipients. Only when the donor cells represented >10% was the recipient considered to be positive. Three and two independent experiments, respectively, were performed for E11 (41–47 somite pairs) and E12 tissues (>60 somite pairs). 1 and 0.33 tissue equivalents transplanted for E11.0 and E12.0, respectively. 2ºE12, secondary transplantation with 3 X 10^6 BM cells isolated from high level repopulated primary recipients that received cells from E12 tissues (two independent experiments). +/+, GATA-2$^{+/+}$; +/-, GATA-2$^{+/-}$.

The Onset of HSC Activity in GATA-2$^{+/-}$ ***Embryos Is Normal.*** Previously, we reported CFU-S_{11} and HSC deficiencies in embryos with a haploid dose of the *runx1* transcription factor (20). The spatial distribution of HSC activity was altered and an unexpected early appearance of HSC activity was found in *runx1*$^{+/-}$ AGM and YS. To examine if there was also a premature appearance of HSC activity in *GATA-2* mutant

embryos, AGM and YS explants from early E10 (31–35 somite pairs) *GATA-2*$^{+/+}$, *GATA-2*$^{+/-}$, and *GATA-2*$^{-/-}$ embryos were isolated, cultured for 3 d, and cells were transplanted into irradiated adult recipients. As shown in **Table II**, although high tissue equivalents (up to five) of cells from *GATA-2* mutant (+/- and -/-) AGM and YS explants were injected, HSC activity was not detected in any of the recipients. Also, *GATA-2*$^{+/-}$ E10.5 YS and E10.5 and E11 FL explants showed no HSC activity. However, HSC activity initiates normally in *GATA-2*$^{+/-}$ AGM explants at E10.5 at the same stage as in the *GATA-2*$^{+/+}$ AGM. HSCs also appear at normal time points in *GATA-2*$^{+/-}$ YS and FL explants (E11 and E11.5, respectively). Therefore, we conclude that HSC induction initiates on schedule and that there is no early onset of HSC activity in *GATA-2*$^{+/-}$ AGM, YS, or FL.

Table II. ***HSC Activity in GATA-2 Mutant Tissues after Explant Culture.***

	AGM explants			YS explants			FL explants	
Stage	+/+	+/-		+/+	+/-		+/+	+/-
E10.5	2[a]/3[b] (66)[c]	1/12 (8)		0/3 (0)	0/11 (0)		ND	0/2 (0)
E11.0	1/2 (50)	0/4 (0)		1/4 (25)	1/5 (20)		0/3 (0)	0/5 (0)
E11.5	11/12 (50)	0/27 (0)		7/25 (28)	7/29 (24)		6/25 (24)	3/28 (11)
E12.0	7/12 (58)	0/13 (0)		4/16 (25)	2/14 (14)		11/12 (92)	10/19 (53)
	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-
E10.0	0/7 (0)	0/8 (0)	0/3 (0)	0/7 (0)	0/8 (0)	0/3 (0)	ND	ND

E10 to E12 AGM, YS, and FL explants were cultured for 3 d, made into a single cell suspension, and injected into irradiated adult recipients. Each result represents [a]the number of recipient mice showing donor cells in the peripheral blood (DNA) isolated at >4 mo posttransplantation, [b]the total number of mice transplanted, and [c]the percentage of repopulated recipients. ND, transplantation not performed. Only when donor contribution was >10% was the recipient considered to be positive. Two to four independent experiments performed for each stage and tissue type. For E10, one to five tissue explant equivalents were transplanted per recipient. 1 tissue explant equivalent was transplanted per recipient for E10.5 and 0.33 tissue explant equivalents for E11.0, E11.5, and E12.0. +/+, GATA-2$^{+/+}$ and +/-, GATA-2$^{+/-}$. E10, 31–35 somite pairs (sp); E10.5, 36–40 sp; E11.0, 41–47 sp; E11.5 >48 sp; E12.0>60sp.

Serial Transplantation Potential of Midgestation HSCs Is Severely Reduced. HSC self-renewal can be tested by serial transplantation of HSCs from primary to secondary recipients. Since we found that *GATA-2*$^{+/-}$ AGM HSCs are severely reduced in their expansion, we examined whether *GATA-2*$^{+/-}$ embryo-derived HSCs are as potent in their serial repopulation ability as wild-type HSCs. Whole BM cells from primary recipient mice showing high donor contribution from transplanted E12 *GATA-2*$^{+/+}$ or *GATA-2*$^{+/-}$

AGM, YS, and FL cells were injected into irradiated secondary adult recipients. Consistent with previous published results, *GATA-2*$^{+/+}$ AGM-, YS-, and FL-derived HSCs can successfully reconstitute secondary recipients; 100% of secondary recipients were repopulated with HSCs from primary recipients of these midgestation tissues (**Table I**). In contrast, HSCs from a primary *GATA-2*$^{+/-}$ AGM recipient failed to repopulate any of the secondary recipients analyzed (0%; zero out of three). Reduced HSC activity was also observed in the secondary recipients receiving BM cells from *GATA-2*$^{+/-}$ YS and FL primary recipients (44% compared with 100% recipient repopulation with *GATA-2*$^{+/+}$ primary BM cells). These results demonstrate that *GATA-2* dose affects HSC serial repopulation ability and suggests a defect in HSC self-renewal.

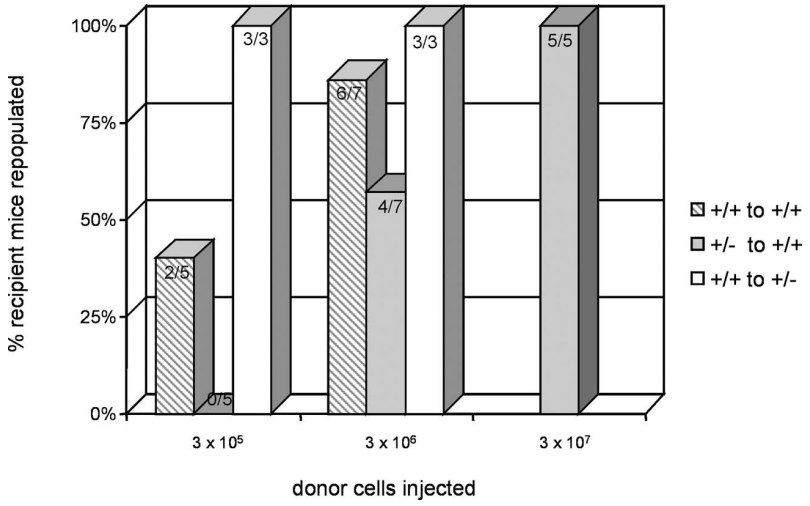

Figure 4. Competitive transplantation of *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ BM in sublethally irradiated adult recipients. Varying concentrations (3 X 10^5–3 X 10^7) of *GATA-2*$^{+/+}$ or *GATA-2*$^{+/-}$ BM cells were transplanted into sublethally irradiated *GATA-2*$^{+/+}$ or *GATA-2*$^{+/-}$ recipients to test for HSC competition in repopulation. The y axis shows the percentage of recipient animals engrafted with >10% donor cells in hematopoietic tissues. Engraftment results are shown in gray striped bars for *GATA-2*$^{+/+}$ donor cells transplanted into *GATA-2*$^{+/+}$ recipients, in gray bars for *GATA-2*$^{+/-}$ donor cells transplanted into *GATA-2*$^{+/+}$ recipients, and in white bars for *GATA-2*$^{+/+}$ donor cells transplanted into *GATA-2*$^{+/-}$. The results show that *GATA-2*$^{+/+}$ HSCs out-compete *GATA-2*$^{+/-}$ HSCs.

GATA-2*$^{+/-}$ *BM HSCs Are at a Competitive Disadvantage. The decreased serial repopulation ability of embryo-derived HSCs prompted us to investigate if adult BM HSCs are also affected by a reduction in *GATA-2* dose. Initially, we injected limiting doses of *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ BM cells into lethally irradiated adult recipients but found no quantitative differences in repopulation. Hence, we performed reciprocal competitive transplantations in which different concentrations of unmanipulated *GATA-*

2$^{+/+}$ and *GATA-2*$^{+/-}$ BM cells were injected into sublethally irradiated *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ adult recipients.

When 3 X 10^5 whole *GATA-2*$^{+/+}$ BM cells were transplanted into *GATA-2*$^{+/-}$ adult recipients, long-term high level donor contribution was found in two out of five (40%) recipients, whereas *GATA-2*$^{+/-}$ cells at this dose provided no repopulation (zero

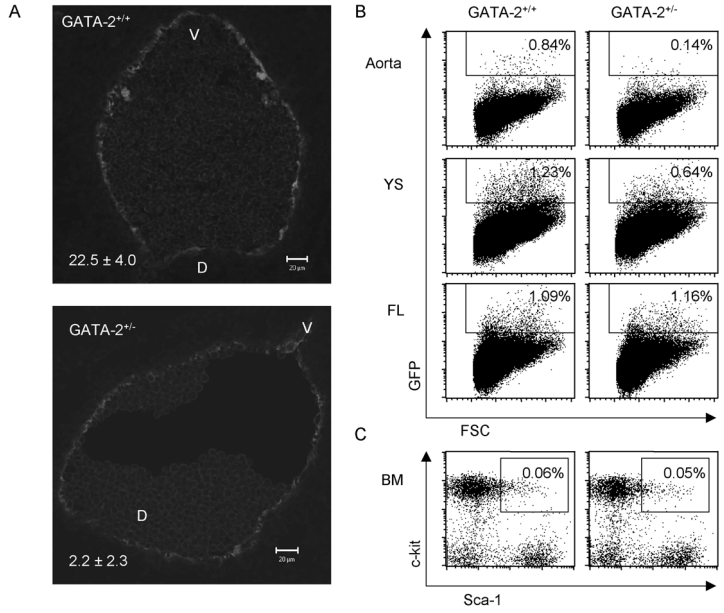

Figure 5. Phenotypic analysis of HSCs in *GATA-2*$^{+/-}$ embryos and adults. *GATA-2–Ly-6A GFP* compound transgenic embryos were generated by timed pluggings. (A) Representative transverse sections through the E11 dorsal aorta of a *GATA-2*$^{+/+}$*–Ly-6A GFP* embryo (45 somite pairs; top) and a *GATA-2*$^{+/-}$*–Ly-6A GFP* embryo (43 somite pairs; bottom). Sections were taken from the caudal end of the AGM, at the height of the hindgut, and stained with anti-CD34 antibody. In total, four embryos were analyzed (2 embryos and a total of 37 sections from each genotype) and cells counted in the aortic endothelium throughout the levels containing the gonads and mesonephroi. $CD34^+$ endothelial cells served as a control for section quality and normalization. Red fluorescence (CD34) and green fluorescence (GFP). The percentage of $GFP^+/CD34^+$ endothelial cells ±SEM is shown on the bottom left and is significantly reduced in the *GATA-2*$^{+/-}$ embryos; $P < 0.05$. Flow cytometric analysis of phenotypically defined HSCs was performed on (B) embryonic hematopoietic tissues and (C) adult BM. Expression of the Ly-6A GFP HSC marker was analyzed on E11 aorta, YS, and FL cells. Adult BM cells were analyzed for the percentage of cells in the Lin^- fraction that are $Sca\text{-}1^+c\text{-}kit^+$. Percentages of GFP^+ cells in the embryonic tissue and $Sca\text{-}1^+c\text{-}kit^+$cells enclosed in each gate are shown.

out of five recipients; 0%) (**Fig. 4**). Only at a 10-fold higher cell dose were the *GATA-2*$^{+/-}$ cells able to repopulate four out of seven (57%) recipients. A dose of 3 X 10^6 *GATA-2*$^{+/+}$ cells repopulated almost all recipients (six out of seven; 86%), whereas 3 X 10^7 *GATA-2*$^{+/-}$ cells were required for repopulation of all recipients (five out of five;

100%). To further examine the competitive abilities of *GATA-2*$^{+/-}$ cells, *GATA-2*$^{+/+}$ cells were injected into sublethally irradiated *GATA-2*$^{+/-}$ recipients. Only 3 X 10^5 (or fewer) wild-type cells were required to fully out-compete all the *GATA-2*$^{+/-}$ HSCs in the recipients. Thus, *GATA-2*$^{+/+}$ HSCs compete more effectively against *GATA-2*$^{+/+}$ HSCs than do *GATA-2*$^{+/-}$ HSCs, demonstrating that *GATA-2*$^{+/-}$ adult BM contains fewer HSCs or that these HSCs are qualitatively less potent.

GATA-2 Dose Affects the Number of Phenotypically Defined HSCs in the Embryo But Not the Adult. Our *in vivo* transplantation results clearly show that *GATA-2*$^{+/-}$ HSC activity is affected throughout development. To more specifically investigate the cell types that are affected in the *GATA-2*$^{+/-}$ mice, we crossed the *GATA-2* mutant allele into *Ly-6A GFP* transgenic mice, in which HSCs can be detected by the expression of the green fluorescent protein (GFP) reporter under the transcriptional control of *Ly-6A* regulatory sequences (26). *Ly-6A* encodes the Sca-1 surface glycoprotein that is expressed on HSCs. Previously, we have shown that all AGM, FL, and adult BM HSCs express the *Ly-6A GFP* transgene and that GFP expression is highly restricted in the AGM region to a few aortic endothelial cells and hematopoietic clusters (23, 27).

To determine whether *GATA-2* dose affects these cells, we examined transverse sections through the E11 dorsal aorta from compound transgenics (*Ly-6A GFP–GATA-2*$^{+/+}$ and *Ly-6A GFP–GATA-2*$^{+/-}$). As shown in representative sections in Fig. 5A, GFP-positive cells are decreased in number in the *GATA-2*$^{+/-}$ aorta compared with the *GATA-2*$^{+/+}$ aorta. Quantitations were performed by counting GFP-positive and CD34-positive cells in 37 aorta sections from each genotype (CD34 immunostaining of endothelial cells provided a normalization control). GFP-positive cells were present but decreased by a factor of 10 or more in the *GATA-2*$^{+/-}$ aortas. Hence, *GATA-2* haploinsufficiency leads to a significant decrease in HSCs and/ or HSC precursors in the AGM.

Flow cytometric analyses were performed to verify these results and to examine if the phenotypic HSC content of the other hematopoietic tissues was also changed. As shown in Fig. 5B, in such compound transgenic embryos a sixfold decrease in GFP-positive cells in the E11 *GATA-2*$^{+/-}$ aorta was found compared with *GATA-2*$^{+/+}$ aorta. E11 *GATA-2*$^{+/-}$ YS showed a 1.9-fold decrease in GFP-positive cells. However, no decrease was found in E11 *GATA-2*$^{+/-}$ FL. Similarly, no difference in the percentages of HSCs as defined by the Lin$^-$Sca-1$^+$c-kit$^+$ (LSK) phenotype was found when *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$adult BM was analyzed (Fig.5C). Together, these phenotypic data support the transplantation data in showing that HSCs are quantatively decreased in the AGM but that HSCs increase to normal numbers in the FL and adult BM.

Cytotoxic Drug Treatment Reveals a Proliferation Defect in GATA-2$^{+/-}$ BM HSCs. To test if the qualitative defect in *GATA-2*$^{+/-}$ HSCs is related to proliferation, *GATA-2*$^{+/+}$

and *GATA-2*$^{+/-}$ mice were treated with the cytotoxic drug 5-FU (28). At 0, 4, 8, 12, and 16 d after treatment, BM cells were tested in *in vitro* assays and analyzed by flow cytometry for evidence of hematopoietic regeneration. As shown in Fig. 6A, both *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ mice showed similar reductions in total BM cell number at 4 d post 5-FU. Total BM cell numbers increased to starting numbers by 16 d post–5-FU. No significant differences were observed between the number of total *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ BM cells at any time point. In addition, no defect in hematopoietic

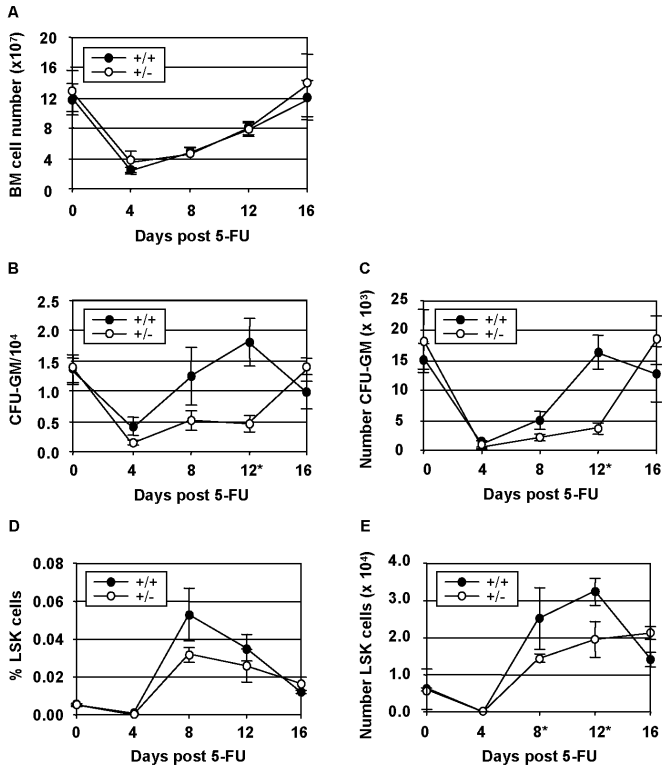

Figure 6. Hematopoietic regeneration after 5-FU treatment. The temporal regeneration of the hematopoietic system within the BM compartment of *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ mice was examined at days 0, 4, 8, 12, and 16 after 5-FU injection for (A) total BM cell numbers, (B) the frequency of CFU-GM in 10^4 BM cells, and (C) the total number of CFU-GM in the BM (two tibias plus two femurs). The regeneration of BM HSCs was examined by quantification of the Sca-1$^+$ and c-kit$^+$ cells within the lin$^-$ population by flow cytometry. (D) Percentage and (E) absolute number of LSK BM cells was examined at days 0, 4, 8, 12, and 16 after 5-FU injection. *GATA-2*$^{+/+}$ samples are represented by ●, and *GATA-2*$^{+/-}$ samples are represented by ○. Two complete time course experiments were performed. Each point represents an average of two to three animals with SEM. *Significant difference between the *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$. (B) CFU-GM frequency day 12, P<0.008. (C) CFU-GM numbers day 12, P<0.002. (E) LSK cell number day 8 and 12, P< 0.04.

differentiation was observed in *GATA-2*$^{+/-}$ BM cells, as flow cytometric analysis showed

the presence of all three lineages, lymphoid, myeloid, and erythroid at similar levels in *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ BM (not depicted).

To investigate whether specific immature hematopoietic progenitors were affected in 5-FU–treated *GATA-2*$^{+/-}$ mice, we performed *in vitro* colony assays for CFU-GM. As shown in Fig. 6, B and C, the starting frequency and number of CFU-GM were the same in *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ BM. At 4 d post 5-FU treatment, frequency and number of CFU-GM reached a similar low point in both *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ BM. However, at 12 d after 5-FU treatment, CFU-GM frequency and number in *GATA-2*$^{+/+}$ BM reached higher or the same levels as the untreated BM, whereas the *GATA-2*$^{+/-}$ BM CFU-GM frequency and number remained low ($P<0.008$ and 0.002, respectively). Only at day 16 after 5-FU treatment did *GATA-2*$^{+/-}$ CFU-GM frequency and number reach the same levels as in untreated BM. Thus, the 4 d delay in the regeneration of CFU-GM in *GATA-2*$^{+/-}$ BM suggests a *GATA-2* dose– related proliferation defect acting within these progenitors. Alternatively, a *GATA-2* dose–related proliferation defect acts within HSCs and only secondarily influences CFU-GM.

To test this, we analyzed the regeneration of HSCs. We performed flow cytometric analysis for LSK BM cells at 0, 4, 8, 12, and 16 d post–5-FU treatment. In both *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ BM, HSC percentages and absolute numbers were similarly reduced at 4 d post–5-FU treatment (Fig. 6, D and E). Both *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ BM HSC percentages and numbers began recovering at day 8 post–5-FU, when they surpassed the initial percentages and numbers. However, *GATA-2*$^{+/-}$ BM HSC numbers remained significantly lower than in *GATA-2*$^{+/+}$ BM at both day 8 and 12 post-5FU-treatment ($p < 0.04$). Furthermore, whereas *GATA-2*$^{+/+}$ HSC numbers peaked at day 12 post-treatment and declined thereafter, *GATA-2*$^{+/-}$ HSC numbers increased slowly up to day 16 post–5-FU treatment. The finding that HSC expansion in 5-FU–treated *GATA-2*$^{+/-}$ mice is delayed by at least 4 d strongly suggests a *GATA-2* dose–dependent proliferation defect in HSCs.

Discussion

The data presented here show for the first time that a full dose of *GATA-2* is required during embryonic and adult stages for quantitatively and qualitatively normal HSC activity *in vivo*. Although HSCs are most likely not produced in *GATA-2*$^{-/-}$ embryos, the effects of *GATA-2* haploinsufficiency had been largely unexplored because such *GATA-2*$^{+/-}$ animals grow normally and present an overtly normal adult hematologic profile. Here we have shown that with only half the dose of *GATA-2*, HSC numbers are severely and specifically reduced in the AGM region, where the first induction and expansion of HSCs is occurring during midgestation. Moreover, AGM HSC quality is compromised. Thereafter, in the other tissues harboring HSCs,

quantitative deficiencies in *GATA-2*$^{+/-}$ HSCs appear to be compensated through normal (albeit delayed) expansion of HSCs, but qualitative deficiencies are retained through to adulthood. Therefore, given that only a few HSCs out of the whole cohort of HSCs are actively contributing to the hematopoietic system at any one time (29, 30) and that *GATA-2*$^{+/-}$ cells are not defective in differentiation, no general hematologic defects would be expected in haploinsufficient adults. Only through stringent *in vivo* transplantations or cytotoxic stress are HSC functional defects observable. The results of these experiments strongly suggest an essential role for GATA-2 in the induction and expansion of the first HSCs in the AGM and an additional, distinctive role for GATA-2 in the proliferation of HSCs.

HSC Quantitative Processes Are Altered in GATA-2*$^{+/-}$ *Mice. In *in vivo* transplantation experiments we show quantitatively reduced HSC activity in *GATA-2*$^{+/-}$ embryos. The four- to ninefold decrease in HSC activity in E11/E12 *GATA-2*$^{+/-}$ AGMs compared with *GATA-2*$^{+/+}$ AGMs is the consequence of fewer HSCs, as aorta sections and flow cytometric analysis show a sixfold decrease in phenotypically defined HSCs. Hence, *GATA-2*$^{+/-}$ AGMs can neither expand nor maintain HSCs compared with *GATA-2*$^{+/+}$ AGMs. In contrast, HSCs are expanded and maintained in E11/E12 *GATA-2*$^{+/-}$ YS (at a slightly decreased number), with the fold decrease in phenotypically defined HSCs in the YS corresponding closely to the fold decrease in HSC activity. Considering the fact that HSCs are first detected in the AGM region and then in the YS and FL, the reduced HSC content of the *GATA-2*$^{+/-}$ YS and FL may well be a secondary effect of the reduction in the *GATA-2*$^{+/-}$ AGM region.

Our *in vivo* analyses for hematopoietic progenitor cells in the AGM region and the YS show that CFU-S_{11} are also *GATA-2* dose dependent. These data are consistent with previous *in vitro* studies on *GATA-2*$^{-/-}$ YS and ES cells (13, 31), showing much reduced hematopoietic progenitor activity. The reduced CFU-S_{11} activity in *GATA-2*$^{+/-}$ AGMs and YSs could further be a consequence of the reduced HSC activity we detected in these tissues. However, the source of cells providing the CFU-S_{11} activity in the embryo is not clear. Whereas in the adult, hematopoietic progenitor cells are derived from HSCs, in the preE10.5 AGM region and the YS they may be derived from hemangioblasts and/or hemogenic endothelium rather than via a HSC ancestor. Hence, GATA-2 may act directly on the *in vivo* generation, survival, and/or expansion of the hematopoietic progenitor cells, HSCs, and/or their direct precursors in the AGM and YS.

The YS as a Compensatory Generator of HSCs Independent of GATA-2 Dose. For over three decades, the origins of adult HSCs have been a focus of research. The view that the mammalian YS is able to provide hematopoietic cells that migrate and colonize the FL and then the BM during the neonatal/adult stages has been altered by the finding

that the first fully functional adult HSCs are autonomously generated in the AGM region. Shortly thereafter, the YS contains HSCs, but due to the experimental constraints of mammalian embryos, it is difficult to definitively demonstrate whether these HSCs are AGM derived or autonomously generated in the YS. Recent data suggest that indeed YS can autonomously generate and expand HSCs (6) and putative pre-HSCs (32). Since we observe a dramatic reduction of HSCs in *GATA-2*$^{+/-}$ AGMs but only a slight reduction of HSCs in *GATA-2*$^{+/-}$ YSs, our transplantation data support the notion of YS HSC generation potential (albeit in a *GATA-2*$^{+/-}$ embryo). However, since HSCs are still generated in the *GATA-2*$^{+/-}$ AGM 1 d earlier than in the YS, it remains possible that YS HSCs are AGM derived. Interestingly, the reduced HSC activity in the *GATA-2*$^{+/-}$ YS can be expanded to a magnitude comparable to that of the *GATA-2*$^{+/+}$ YS in explant cultures, suggesting that at least some of the reduced activity in the *GATA-2*$^{+/-}$ YS is a secondary effect of the reduction of HSCs in the AGM. Furthermore, *GATA-2*$^{+/-}$ HSC numbers are compensated to normal levels in the adult, possibly due to HSC generation and expansion in the YS and the further expansion in the FL and BM. Notwithstanding, these data imply that the underlying molecular mechanisms in which the AGM generates, maintains, and expands HSCs are different from that of the YS. The AGM region is exquisitely sensitive to the level of the *GATA-2* dose, whereas the YS is much less sensitive. Hence, the HSC defects in *GATA-2*$^{+/-}$ AGMs do not result in severe anemia in adults since *GATA-2*$^{+/-}$ YS can generate and/or expand sufficient HSCs irrespective of the haploinsufficiency.

HSC Qualitative Processes Are Altered in GATA-2$^{+/-}$ ***Mice.*** The results of adult BM competitive transplantation experiments clearly demonstrate a qualitative difference between the *GATA-2*$^{+/+}$ and *GATA-2*$^{+/-}$ HSCs. The high percentage of *GATA-2*$^{+/-}$ mice engrafted with *GATA-2*$^{+/+}$ cells, even at low donor cell numbers, demonstrate that *GATA-2*$^{+/+}$ BM HSCs have a proliferative advantage over the *GATA-2*$^{+/-}$ BM HSCs. In the reciprocal transplantation in which *GATA-2*$^{+/-}$ BM cells were transplanted into *GATA-2*$^{+/+}$ recipients, high numbers of cells were needed to obtain a high percentage of donor-engrafted recipient mice and thus imply that: (a) the number of HSCs in *GATA-2*$^{+/-}$ BM is quantitatively reduced; (b) the *GATA-2*$^{+/-}$ HSCs have a lower proliferative advantage over the *GATA-2*$^{+/+}$ HSCs; and/or (c) the homing efficiency is lower for *GATA-2*$^{+/-}$–derived HSCs. The fact that no significant difference in the percentage or absolute number of LSK BM cells was found between *GATA-2*$^{+/-}$ and wild-type BM indicates that the decreased HSC activity is not due to a quantitative decrease in *GATA-2*$^{+/-}$ BM HSCs. However, the delayed expansion of HSCs in the 5-FU recovery experiments does strongly suggest that the major GATA-2 dose-dependent defect is in HSC proliferation. Although homing of HSCs is not required in this experimental scenario, we cannot exclude an additional GATA-2 dose–dependent defect in homing.

How Does GATA-2 Dose Affect the Quantitative and Qualitative Development of HSCs? We propose here that the *GATA-2* dose effects we observe in the AGM act at the level of the hemogenic cells that differentiate into HSCs. Normally, a full dose of *GATA-2* is required for the generation, maintenance, and/or expansion of these precursor cells. In the haploinsufficient AGM region, these hemogenic cells fail to differentiate, survive, and/or divide. However, owing to the stochastic nature of gene expression, some hemogenic cells still achieve a threshold level of GATA-2 protein, and therefore, the target genes (which are needed for the differentiation, survival and/or division of the precursor cells) can be activated at some low frequency in the E10.5 AGM. The outcome of *GATA-2* haploinsufficiency is then a small production of HSCs followed by an overall reduction in the absolute number of AGM HSCs that we can detect functionally in our transplantation assay and phenotypically in immunostained sections and flow cytometry analysis.

Recent GATA-2 expression data in the AGM support the notion of a role for GATA-2 in hemogenic precursors. Transgenic embryos with a GFP marker under the control of *GATA-2* transcriptional regulatory sequences show high levels of GATA-2 expression in $CD45^-$ AGM cells with hemogenic potential and a significant decrease in the percentage of $CD45^+$ cells in *GATA-2*$^{+/-}$ E11.5 AGMs (12). Moreover, during midgestation, at the time of the first induction of HSCs, GATA-2 is expressed in the endothelial cells lining the dorsal aorta and some underlying mesenchymal cells. Hence, high GATA-2 expression in hemogenic cells of the AGM suggests that GATA-2 is acting on the cells just immediately preceding the induction of HSCs.

Since GATA-2 is a transcription factor, its target genes within hemogenic AGM cells are of particular interest. Several markers of AGM HSCs and aortic hemogenic cells have been recently described: the Ly-6A (Sca-1) cell surface glycoprotein (23, 33) and Runx-1 transcription factor (27). These molecules are overlapping with GATA-2 in their expression patterns in hemogenic cells of the dorsal aorta. Targeted mutation of these genes results in qualitative and/ or quantitative defects in HSCs. Whereas *Ly-6A*$^{-/-}$ embryos thrive into adulthood with no or little effect on HSC generation in the embryo, functional analyses of HSCs derived from *Ly-6A*$^{-/-}$ mutant BM show defects in their self-renewal ability (34), similar to our findings in *GATA-2*$^{+/-}$ BM. In contrast, *runx-1*$^{-/-}$ embryos are completely devoid of HSCs and exhibit FL anemia leading to lethality at E12 (35–37). Moreover, *runx-1* haploinsufficiency leads to a premature extinction of AGM HSCs (20). Hence, the *Ly-6A* and *runx-1* genes could be targets of GATA-2 or, alternatively, contribute to the activation of the same pathways for HSC self-renewal and/or HSC generation. At present, although many GATA consensus-binding sites appear in the sequences surrounding these genes, there is no *in vivo* data showing that any of these sites are functional.

Nonetheless, two bona fide target genes of GATA-2 have been proposed. These are SCL/tal-1, an essential early hematopoietic transcription factor and E4bp4, a transcription factor implicated in cell survival. *In vivo* studies show that GATA-2 forms a multiprotein complex with Fli-1 and Elf-1 that binds the *SCL* enhancer and activates the expression in HSCs, endothelial cells, and their bipotent progenitor, the hemangioblast (38). However, *in vivo* mutation analysis on the HSC-specific GATA sites within the *SCL* locus affects SCL expression not only in the AGM but also in YS and FL (38). Therefore, it is unlikely that the selective defect in the *GATA-2*$^{+/-}$ AGM HSCs can be attributed to defective SCL expression. Chromatin immunoprecipitation studies on BaF3 cell line stimulated with IL-3 show that GATA-2 binds to a sequence downstream of the transcriptional start site of E4bp4 and is necessary for transcriptional activation of this gene (39). Considering that IL-3 is a survival factor for HSCs, it is plausible that GATA-2 is involved in the activation of this pathway.

In conclusion, GATA-2 dosage is important in regulation of HSC production and expansion. Haploinsufficiency of *GATA-2* results in quantitative decreases in HSCs in the AGM and qualitative defects in HSCs in both the embryonic-derived and adult BM HSCs. The pivotal importance of GATA-2 in these processes within HSCs now awaits the identification of the relevant target genes and the functional cascades that GATA-2 activates, most likely in concert with other factors in multiprotein complexes.

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CHAPTER 5

General Discussion

General Discussion

Hematopoiesis can be considered not only as a homeostatic process in which different morphologic and functional types of mature blood cells are produced, but also as a complex process of hematopoietic fate determination, proliferation and cell survival during its development in the embryo. Hematopoietic cells are regulated at the molecular level either by intrinsic and extrinsic signals. Beginning with its emergence in the embryo, the hematopoietic system resides in a microenvironment of interacting cells and factors. Thus, extensive studies in the multilayered regulation and cross talk required for hematopoietic (stem) cell emergence are being intensively studying.

In addition, the hematopoietic system is highly dynamic during ontogeny, emerging and residing in several different anatomical sites. Different microenvironments during embryonic, fetal and adult stages influence whether multipotent hematopoietic cells are generated, expanded, maintained or induced to migrate. Hematopoietic fate determination occurs in at least two distinct tissues in the embryo, the yolk sac and the AGM. However, the precise embryonic origin of the HSCs found in the adult bone marrow of mammalian species still remains undefined. Many approaches have been taken to delineate the developmental origin(s) of the hematopoietic system using various species of vertebrates. Briefly, in non-mammalian vertebrates it has been demonstrated that the adult hematopoietic system has an intraembryonic origin. Fate mapping studies strongly suggest an aortic endothelial origin for definitive HSCs. In the mouse, the AGM is the only site where autonomous and independent production of definitive HSCs has been proven. However, whether these adult type of HSCs from the mammalian AGM seed the adult hematopoietic sites still awaits lineage tracing experiments.

GATA-2: involvement in HSC development

Thus far, distinct hypotheses have been proposed concerning the origin(s) of the mammalian hematopoietic system and its most potent cell type, the HSC. There are at least two (and probably more) distinct sites in which cells can take on hematopoietic fate, including sites in the embryo proper and extraembryonic sites. Interestingly, within these sites there have been found various possible HSC precursors, but their direct relationship to HSC is not yet established. Among these precursors, the most plausible are hemogenic endothelial cells and hemangioblasts, although the existence of the latter within the *in vivo* embryo body still requires further insight. In addition, the molecular program or mechanism of the HSC generation with respect to an endothelial origin remains unclear. An interesting transcription factor that may be implicated at early stages of HSC development and perhaps in the regulation of hemogenic endothelium is GATA-2. GATA-2 was found to be expressed in cells with HSC phenotype in the aortic endothelium and in the cells of the emerging hematopoietic clusters. This expression

coincides with the detection of functional HSCs, suggesting that GATA-2 may act by specifying HSC fate. However, the early expression of GATA-2 at E7.7 in the lateral and extraembryonic mesoderm (Minegishi et al., 1999; Minegishi et al., 2003 does not coincide with HSC activity and thus, although required for midgestation AGM HSC activity, GATA-2 most likely does not act alone.

More quantitative analysis has shown that GATA-2 is highly expressed in $CD45^-$ subpopulation of E11.5 AGM, which include cells expressing markers of hemogenic precursors such as CD34 and PCLP1 (Minegishi et al., 2003). Furthermore, overlapping expression of Sca-1, Runx1 and GATA-2 in the vascular endothelium of the dorsal aorta at midgestation suggests even more strongly that GATA-2 acts together with Runx1 (Yokomizo et al., 2001). Together they may play a role in transition of hemogenic endothelium to the hematopoietic cell or be involved in hemangioblast formation. Using a GFP reporter, where GFP was placed under control of the early hematopoietic regulatory domain of the *GATA-2* gene, GFP^+ cells were localized within the E9.5 P-Sp region. When these cells were placed in an *in vitro* co-culture system in the presence of VEGF they were able to differentiate into both endothelial and hematopoietic cells. This was not the case for E9.5 YS cells (Kobayashi-Osaki et al., 2005). Thus, the P-Sp/AGM most likely contains hemangioblast-like cells expressing GATA-2. However, it needs still to be elucidated what is the molecular mechanism of GATA-2 action in these HSC precursors. An interesting finding in delineating the transcriptional network in HSC formation is the fact that one of the target genes of GATA-2 is *SCL* (Göttgens et al., 2002). SCL has been shown to be essential in the development of embryonic HSCs, including their possible precursors. Also other genes such as *Bcl-xL*, *E4bp4*, *Evi-1* (Yuasa et al., 2005) implicated in regulation of HSC activity or cell survival (Rodrigues et al., 2005; Yu et al., 2002) could be considered as possible transcriptional cofactors, acting either downstream or upstream (*Evi-1*) through GATA-2 in regulation of HSC development.

A recently proposed scenario for definitive HSC generation involves the migration of HSC precursors from subaortic patches within the ventrolateral part of the dorsal aorta. During their migration from the subaortic patches into the mesenchymal region underlying the dorsal aorta, the precursors eventually protrude through endothelium to contribute to the hematopoietic clusters. As they finally enter the lumen of the dorsal aorta they become functional HSCs (Bertrand et al., 2005; Jaffredo et al., 2005). It remains to be determined through single cell marking and functional analysis whether hemangioblasts or hemogenic endothelial cells are related to each other and whether either of them represents the precise direct precursor cell to HSCs (Park et al., 2005).

Regarding the *in vivo* presence of hemangioblasts, apart from the YS, hemangioblast activity was also detected in the embryo body (Huber et al., 2004; Jaffredo et al., 2005). Genetically marked mesodermal precursors were shown to express Flk-1 and SCL. Both of these molecules have been shown by gene targeting experiments to play an essential role in hemangioblast generation (Shalaby et al., 1995;

Robb et al., 1995). Thus, hemangioblasts, based at least on their anatomical localization, could be classified into the primitive and definitive types. To date, the detection of hemangioblasts or hemogenic endothelial cells in the embryonic placenta has not been reported. However, endothelial cells lining the major vasculature of the midgestational placenta express GATA-2 and Sca-1 (Ottersbach and Dzierzak, 2005). Analysis of Ly-6A (Sca-1) GFP transgenic embryonic placenta has shown that all functional HSCs reside within the GFP^+ population of cells. Thus, it is highly likely that GATA-2 also functions in placental HSCs.

GATA-2 not only functions during HSC development in precursors to HSCs, but as already mentioned in **Chapter 1,** GATA-2 functions during early stages of hematopoietic cell differentiation. Since it was known that a complete lack of GATA-2 affects all definitive hematopoietic lineages, we have shown in **Chapter 4** that even a haploinsufficiency of *GATA-2* affects the activity of multipotent hematopoietic cells. This dose-dependent effect of *GATA-2* was very pronounced, resulting in a defective production and expansion of AGM HSCs at midgestation. Transplantations of *GATA-2* $^{+/-}$ E11 or E12 AGM into irradiated adult recipients have shown 4 to 8-fold reduction in the percentage of repopulated mice, whereas the HSC repopulating activity of YS and FL at this stage was either slightly affected (for YS) or not at all affected (for FL). Moreover, data obtained from secondary transplantations of *GATA-2*$^{+/-}$ E12 AGM have shown a very severe defect in the self-renewal of these HSCs, since no engraftment was observed.

Additionally, in order to test the *GATA-2*-dose effect on the expansion of HSCs *in vitro*, E10-E12 AGM, YS and FL tissues from *GATA-2*$^{+/-}$ embryos were explanted for 3 days, followed by transplantation. HSC activity was significantly decreased for E10.5 *GATA-2*$^{+/-}$ AGM (8-fold compared to *GATA-2*$^{+/+}$). Also, HSC activity was extinguished in E11 and E12 *GATA-2*$^{+/-}$ AGMs. Thus, the single dose of *GATA-2* gene is not sufficient for the normal emergence or expansion of functional AGM HSCs. In addition, activity of hematopoietic multipotent progenitors (CFU-S) was highly decreased for explanted E10.5 AGM$^{+/-}$ compared to wild type AGM, indicating that GATA-2 levels not only have an impact on the HSCs but also on AGM multipotent progenitors. Whether this is a direct effect on CFU-S or an indirect effect through the HSC or preCFU-S is undetermined. In contrast, no severe defects of GATA-2 haploinsufficiency were observed for YS or FL HSCs, underlining the importance of both copies of the *GATA-2* gene for the generation and expansion of AGM HSCs. This dose-effect is somewhat reminiscent of but not identical to *Runx1* dose-related HSC defects. In the case of *Runx1* haploinsufficiency, HSCs appeared prematurely in the AGM and YS. In *GATA-2* haploinsufficient embryos, HSCs were never found prematurely in YS. Interestingly, slightly reduced hematopoietic progenitor activity and repopulating HSC activity were found, suggesting that *GATA-2* dose is important for maintaining the HSC activity in the YS. Considering that *GATA-2* haploinsufficiency perturbs multipotent hematopoietic cells in the AGM and YS, it is possible that such

perturbations also occur in placental HSCs. Hence, future studies will focus on the role of GATA-2 and other factors in the placenta.

Since GATA-2 represents one of the essential regulators of HSC potential during embryogenesis, we were interested if the same *GATA-2* dose-dependent effect could be found in HSCs in the adult. The HSC defect in *GATA-2*$^{+/-}$ BM HSCs appears to be more qualitative, since the number of LSK cells from Ly-6A GFP transgenic adult BM was comparable between wild type and *GATA-2* heterozygous mutant mice. However, with the additional use of the CD34 marker, others found a decreased frequency of hematopoietic stem/progenitor cells in *GATA-2*$^{+/-}$ BM (Rodrigues et al., 2005). By studying hematopoietic activity *in vitro* a significant reduction in CFU-GM colonies but not other types of colonies (such as erythroid) was observed in *GATA-2*$^{+/-}$ BM cells. Previously it has been shown indeed that GATA-2 can affect the GM pathway (Heyworth et al., 1999) or that a haploid dose of *GATA-2* may result in a quantitative defect in production of myeloid progenitors. Interestingly, as we have shown in **Chapter 4**, 5FU- treatment of adult *GATA-2*$^{+/-}$ mice, followed by temporal analysis of BM HSC regeneration in parallel with wild type BM HSCs revealed a proliferative defect. CFU-C analysis also showed a decrease in a number of GM colonies after 5FU-treatment of *GATA-2*$^{+/-}$ adults, corresponding well to the recent results of Rodrigues et al. (2005). In contrast to our findings, the results published by Rodrigues et al., suggest that the haploinsufficiency of GATA-2 does not affect self-renewal of adult repopulating BM HSCs, but rather is related to increased level of apoptosis and reduced HSC cycling. Based on our experimental settings, using distinct competitive transplantation assays and following *GATA-2*$^{+/-}$ BM HSC regeneration for longer time (analysis 1 day after 5FU-treatment done by Rodrigues et al.), it is difficult to compare both of the data directly. However, from both studies it can be concluded that *GATA-2*$^{+/-}$ BM HSCs perform poorly in competitive transplantations.

Neonatal repopulating cells

Neonatal repopulating cells have been suggested to be a type of HSC precursor since they possess only some characteristics of definitive HSCs. They are able to self-renew and provide a multilineage engraftment of newborn recipients, although their proliferative potential is limited and never reaches a very high level of chimerism. As possible HSC precursors, they appear earlier than E10, before the appearance of adult type of HSCs (Müller et al., 1994). By definition, these multipotent hematopoietic cells are capable of repopulating the hematopoietic system of neonates, but they fail to engraft adult recipients. This remarkable difference in repopulation was proposed to result from expression of distinct adhesion/or homing molecules between adult and neonatal hematopoietic microenvironment - the neonatal microenvironment is similar to that of the liver during fetal stages (Lu et al., 1996; Yoder et al., 1997a; Yoder et al., 1997b). Interestingly, using already mentioned W mutant neonates (**Chapters 1** and **3**)

it has been shown recently that one of the factors regulating the seeding of donor HSCs in neonatal BM niche is STAT5 (the signal transducer and activator of transcription) (Couldrey et al., 2005). STAT5-deficient BM-derived repopulating cells appeared to be 10-fold more defective in long-term repopulation of neonates than wild type neonatal BM HSCs. Thus, STAT5 may represent one of the important neonatal microenviromental factors required for successful engraftment.

Another selective advantage affecting the repopulation of neonatal HSCs is the sustained hematopoietic activity of the liver and the not yet developed immune system. Thus, to study the activity of embryonic HSCs *in vivo*, the fetal and neonatal microenvironments provide the most optimal conditions for engraftment. Hence, selecting a suitable type of recipient in HSC functional assays is one of the most important experimental parameters affecting not only the ability to home to the proper hematopoietic microenvironment, but also affecting the level of hematopoietic repopulation.

To obtain engraftment by preE10 HSCs or increase a percentage of repopulated mice or engraftment level in individual recipients there have been developed various neonatal transplantation protocols. The most often used are conditioning through irradiation of neonates or busulfan treatment of pregnant dams to ablate or decrease the endogenous pool of HSCs or hematopoietic progenitors. However, such conditioning or busulfan administration regimes for certain strains of newborn can be very harmful and can affect the survival of neonatal recipients. In addition, the type of injection used for cell transplantation appears to be also a risk factor for the survival efficiency and engraftment. In transplantation studies where E8-E10 YS cells were injected through uterine wall of pregnant females, although donor-derived YS cells were detected in transplanted neonatal recipients, the level of repopulation was very low and not consistent (Weissman et al., 1978). Furthermore, such *in utero* transplantation was also performed to test neonatal repopulating activity of E9 YS or PB cells (Toles et al., 1989). While high level chimerism was obtained in individual recipients, the percentage of repopulated newborns remained very low due to the loss of the majority of injected fetuses. Thus, intrahepatic or intravenous injections are safer and more efficient (Yoder et al., 1997a; Yoder at al., 1997b). Using busulfan-conditioned wild type neonates for transplantation of E9 or E10 YS or P-Sp resulted in a long-term, multilineage reconstitution (Yoder and Hiatt, 1997; Yoder et al., 1997a; Yoder at al., 1997b). With regard to HSC generation, in this experimental set up it was not examined if these neonatal repopulating HSCs are produced *de novo* in the YS or P-Sp region, since the embryonic circulation is established at E8.5. However, from other studies using alymphoid (Cumano et al., 2001) adult or normal adult recipients it has been shown that both E8 YS and P-Sp (upon co-culture with BM stromal or AGM-derived endothelial-like cells) contain hematopoietic cells capable of repopulating the adult recipients (Cumano et al., 2001; Matsuoka et al., 2001). However, chimerism was less than 1%. Whereas E8 YS-derived repopulating cells provided only short-term myeloid repopulation, the E8 P-Sp contributed both to myeloid and lymphoid lineages, thus

supporting the intraembryonic origin of definitive HSCs (Cumano et al., 2001). Hence, multipotent hematopoietic cells capable of repopulating neonatal or alymphoid adult recipients were found to be generated in predecessor tissue of AGM, P-Sp region but their proliferative potential appeared to be very restricted compared to the adult repopulating HSCs detected in E10.5 AGM region (Müller et al., 1994; Medvinsky and Dzierzak, 1996).

As previously mentioned, studying neonatal HSC activity *in vivo* does not necessarily require the conditioning of newborn recipients. The already described W mutant, W^{41}, has been used in our studies. Conditioning of W^{41} neonates by irradiation is not advisable, since these mice are very sensitive to irradiation. However, their HSCs are poorly competitive compared to wild type HSCs (Harrison et al., 1980; Barker et al., 1988). Considering our data presented in **Chapter 3**, the frequency of repopulated W^{41}/W^{41} unconditioned newborn recipients is much lower than of published frequency of repopulation of busulfan-treated neonates. This low frequency of engraftment of W^{41}/W^{41} could reflect differences between an unperturbed microenvironment and a chemically conditioned microenvironment. Additionally, while endogenous W^{41}/W^{41} hematopoietic stem/progenitor cells are defective, they most likely still compete with the donor cells for microenvironmental niches. To increase the frequency of engraftment, future studies should include transplantation into busulfan conditioned W^{41}/W^{41} neonates.

Our studies have shown that E9-E10 hematopoietic tissues isolated from *in utero* developed embryos are capable of engrafting neonatal W^{41}/W^{41} recipients. Importantly, our studies have shown for the first time that E10 YS and AGM cells from *in vitro* cultured embryos contain neonatal repopulating cells capable of self-renewal. The significance of this result lies in the fact that whole embryos can only be cultured until the 35-somite pair stage. It is only at this stage that the first adult repopulating HSCs begin to be detected. Hence, the use of whole embryo cultures for studies of fate mapping exclude (at least for the present time) the possibility of lineage tracing these fully potent HSCs. However, we have shown that it is possible to detect neonatal repopulating HSCs. Surprisingly, the frequency of W^{41}/W^{41} neonates repopulated with hematopoietic tissues from *ex utero* developed embryos is higher that from freshly isolated tissues. Future studies will focus on the tracing the origins of these cells in whole *ex utero* cultured embryos.

Following HSC from the embryo to the adult

The lineage relationships between mammalian embryonic HSCs and putative direct precursors and cells of the adult hematopoietic system still remain unclear. Thus, to trace the hematopoietic (stem) cells, different classical or more advanced fate mapping methods have been attempted. Classical lineage tracing with fluorescent dyes or immunochemical markers can be useful only for a short-term marking of cells. In

contrast, labelling of cells through molecular recombination is a permanent and more reliable method to follow rare cells such as HSCs from embryonic/fetal until adult stages. We applied the already well-known Cre/loxP recombination system in our studies. The two most important aspects in obtaining the efficient and reliable marking of multipotent hematopoietic cells are the tissue-specific expression of Cre recombinase and the temporal control of Cre-mediated recombination events. Thus, to control Cre-mediated recombination in a spatio-temporal manner in hematopoietic system, we have used Tamoxifen-inducible Cre recombinase. The expression was driven by regulatory elements of *Ly-6A* gene, encoding cell-surface marker expressed on by all embryonic and adult HSCs (Ma et al., 2002a; Ma et al., 2002b; Spangrude and Brooks, 1993).

To be able to trace HSCs from the embryo to the adult we decided to genetically mark HSCs either at the time of their emergence in the AGM or the time of high HSC activity (in the AGM or FL) to increase the probability of recombination in HSCs. However, using the different Tamoxifen-treatment protocols (regarding the dose and number of injections) and various embryonic stages of induction of Ly-6A CreERT-mediated recombination, we were not able to efficiently mark HSCs or hematopoietic progenitors. The very low efficiency of recombination in our studies appeared to be one of the main drawbacks. Although Cre was found to be expressed in the hematopoietic tissues and HSCs where Ly-6A is normally expressed, rare recombination/expression events were observed. This could be due to structural disadvantages of the CreERT fusion protein we have used. This type of CreERT contains the murine LBD of the mouse ER (estrogen receptor) with only a single mutation (G525R). To date more Tamoxifen-inducible Cre recombinases have been generated. These contain not only more mutations in LBD, but also modifications of the *Cre* gene. The introduction of more than one amino acid substitution in the human LBD appears to improve efficiency of Cre-mediated recombination. For example, CreERT1 and CreERT2 with triple mutations within the human LBD (Feil et al., 1997; Indra et al., 1999; Imai et al., 2001) appear to be very sensitive to low concentration (nanomolar) of 4OHT (Feil et al., 1997). Furthermore, CreERT2 requires lower Tamoxifen/or 4OHT concentrations than CreERT1. Additionally, the translocation of CreERT2 to nucleus occurs much more rapidly (Indra et al., 1999). Thus, the triple amino acid mutation in human LBD most likely increases the affinity of ligand binding and allows a more efficient translocation of Cre into the nucleus to perform recombination.

An additional strategy to enhance recombination efficiency was to genetically modify Cre. These modifications include reduced CpG content, improved Kozak consensus sequence and introduction of silent mutations, which resulted in a new improved form of Cre recombinase. Surprisingly, although this iCre (improved Cre) protein was expressed 1.6-fold higher compared to standard Cre, it was only 1.8-fold more efficient in performing recombination (Shimshek et al., 2002). Thus, such molecular modifications did not significantly increase the recombination efficiency. Moreover, an inducible variant of iCre recombinase, containing on both N- and C-terminus ERT2 domains was generated and tested. Although this recombinase showed 2

times higher activity *in vitro* (Zhang et al., 1996), when applied *in vivo* under the control of a brain-specific promoter, it resulted in only 5-10% recombination compared to 50% recombination obtained in mice expressing Cre constitutively (Casanova et al., 2002). Hence, modifications of Cre and LBD have improved the efficiency of recombination to some extent, but are highly dependent upon other variables such as Tamoxifen/or 4OHT dose, administration route and frequency of treatment to enhance level of Cre-mediated recombination.

In vitro 4OHT-dose-dependent experiments show that already within 2 days, at the lowest 4OHT concentration (10pM), 75% of cultured embryonic fibroblasts were recombined, demonstrating high efficiency (Hayashi and McMahon, 2002). In contrast, with regard to our own data, uncontrollable local variation of Tamoxifen/or 4OHT concentrations *in vivo* most likely decreased recombination efficiency. In addition to ligand concentration variation, it is also known that particular lox reporter lines vary in their ability to be recombined and/or express the reporter gene. Both of the reporter lines, namely Rosa26 lox lacZ (Soriano et al., 1999) and Rosa26 lox EGFP (Mao et al., 2001) used in our Cre/loxP system appear to have some disadvantages. When we used the Rosa26 lox lacZ reporter line (**Chapter 2**), high lacZ staining backgrounds in hematopoietic cells interfered with the detection of bona fide recombined/lacZ expressing hematopoietic cells. In addition, *in vitro* recombination data of others showed a low level of lacZ activity in the absence of inducible ligand (Zhang et al., 1996; Feil et al., 1997). It was speculated that this was due to the proteolysis of the Cre-LBD fusion protein (Zhang et al., 1996). In the case of the Rosa26 lox EGFP line, only a low intensity fluorescent signal in recombined cells was detected. Also, Mao et al. (Mao et al., 2001) show that in GATA-1 Cre: Rosa26 lox EGFP mice, fetal and adult erythroid cells from some hematopoietic organs failed to express EGFP. However, all lymphoid and myeloid lineages have shown to express detectable EGFP as a result of lymphoid and myeloid specific Cre-mediated recombination (Mao et al., 2001). Thus, application of other available lox reporter lines such as Rosa26 lox EYFP (Srinivas et al., 2001) or PGK lox EGFP (Gilchrist et al., 2003), which both showed a high intensity of fluorescence, should improve the efficiency of lineage tracing of hematopoietic cells.

Another interesting strategy to label a discrete group of hematopoietic cells would be to use tetracycline (tet)-responsive system, where temporal expression of Cre recombinase is controlled at the transcriptional level. In contrast to Cre/loxP technology, to control recombination activity of Cre in tet-responsive system, two components are required - a transactivator gene encoding fusion protein called tTA and a tetO recombinase transgene. In one of the variants of this system, named tet-on, tTA protein only in the absence of the ligand binds to tet operon to activate the transcription of the Cre transgene. Thus, to switch off Cre expression temporal administration of tet/or dox (doxacycline- tet analog) is needed (Furth et al., 1994; Branda and Dymecki, 2004). However, to induce Cre expression, followed by induction of recombination only in the presence of ligand, the so-called tet-off was developed, including reversed transactivator gene (rtTA). Although the tet-off system used in controlling Cre-

mediated recombination does not require the continuous administration of tet or dox (as does the tet-on system), *in vivo* studies showed variable kinetics of rtTA in induction of Cre expression and thus recombination. While using rtTA-based system in the presence of dox, Cre expression was detected very rapidly (within 1hr after dox-treatment) in the adults (Hasan et al., 2001), in embryos it was after 13 hrs post treatment (Shin et al., 1999). Hence, considering the very long periods of time needed for activation of Cre recombinase and the additional time required for induction of recombination gene expression, this tet-responsive system seems to be not suitable for following the fate of HSCs during development. Moreover, generation of triple transgenic mice to test Cre-mediated recombination and potential Cre leakiness (affected by integration site of tetO Cre transgene into the genome) create many complications. Nevertheless, the hematopoietic progenitor specific regulatory elements of the *CD34* human gene were used to drive expression of tTA gene in mice (Radomska et al., 2002). The mice expressing CD34-tTA were crossed with tetO Cre transgenics, and in the presence of tet some recombination was observed in adult hematopoietic cells. However, no HSC tracing experiments were performed. Collectively, application of tet-responsive system in fate mapping of multipotent hematopoietic cells represents a potential alternative system of controlling Cre recombination activity in hematopoietic system and will require further modifications such as rapid gene activation of Cre and recombination reporter.

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CHAPTER 6

Summary

Summary

During embryonic and adult stages, the development of hematopoietic cells occurs in distinct, highly vascularized anatomical sites. In the embryo, these sites include the yolk sac, AGM, liver and placenta. In the adult, hematopoiesis occurs mainly in the bone marrow. Although hematopoietic hierarchy is not strictly conserved between the embryo and the adult, the most potent cell type for both hematopoietic systems is HSC. This cell, characterized by high nuclear to cytoplasmic ratio and the expression of specific molecular markers, is capable of self-renewing and also giving rise to morphologically and functionally distinct hematopoietic cells. Following the differentiation of HSCs, mature hematopoietic cells take part in one of many functions such as transport of essential gases (erythroid), destruction of invading cells or organisms (myeloid) or eliciting immune responses to pathogens (lymphoid).

Hematopoietic lineage fate decisions by HSCs are thought to be regulated not only by dynamic changes in the microenvironment but also by HSC-intrinsic factors. The molecular nature of the intrinsic regulation of HSC emergence or activity remains intensely investigated. One of the transcription factors crucial for HSC development is GATA-2. It was previously demonstrated that a lack of this transcription factor affects HSC activity in the embryo. We found in the studies presented in this thesis that *GATA-2* levels, such as a diploid dose of the factor, are also important for HSCs. To study the effect of *GATA-2* dose on HSCs in both adult and embryonic hematopoietic compartments, we performed a series of *in vitro* and *in vivo* assays. A haploid dose of *GATA-2* particularly affected HSCs in the AGM, the site where the first definitive HSCs are autonomously generated in the embryo (at E10.5). We observed a severely reduced production and expansion of midgestational *GATA-2*$^{+/-}$ AGM HSCs under both *in vitro* and *in vivo* conditions. HSC activity in the YS was only slightly affected, while FL HSCs generally appeared unaffected. However, in serial and competitive transplantations, all the fetal and adult *GATA-2*$^{+/-}$ HSCs showed a qualitative defect. In addition, a cytotoxic treatment of *GATA-2*$^{+/-}$ adults affected the proliferative potential of BM HSCs. Thus, our data clearly indicate that GATA-2 acts in a dose-dependent manner in the growth and expansion of HSCs throughout ontogeny.

In addition to studying the molecular program implicated in HSC regulation, an important aspect of HSC development involves defining the precise cellular precursors of definitive HSCs. Fate mapping experiments in chick embryos have identified endothelial cells as direct precursors to hematopoietic cells. The existence of hemogenic endothelial cells was further strongly supported by *in vitro* and histological studies. However, in the murine system, a direct lineage relationship between vascular endothelial and hematopoietic stem cells has not been demonstrated. Some investigators have proposed that direct precursors of HSCs could be already committed multipotent hematopoietic progenitors that can repopulate neonatal mouse recipients but not adult recipients. Such neonatal repopulating cells are detected in the YS and P-Sp (predecessor tissue of AGM) before emergence of first adult repopulating HSCs in the

AGM region. Phenotypic and functional characterization of these multipotent cells has shown that they express HSC cell-surface markers. Our data presented in this thesis provide additional insights on the hematopoietic activity of neonatal repopulating cells. With an eye towards using whole cultured mouse embryos in fate mapping experiments, we used unconditioned (without myeloablative treatment) neonatal hematopoietic mutant W41/W41 (c-kit mutant) mice as recipients for the analysis of early embryo hematopoietic activity. Indeed, transplantation of preE10 P-Sp and YS, isolated from either *in utero* or *in vitro* developed (genetically marked) embryos, into unconditioned W^{41}/W^{41} newborns resulted in a long-term, multilineage donor-cell repopulation. Hence, such multipotent neonatal repopulating cells developed both *in utero* and under our *in vitro* whole embryo culture conditions. With regard to large technical difficulties in the manipulation of mouse embryos developing *in utero*, *in vitro* culture of whole murine embryos is a very attractive method allowing for the proper development of neonatal repopulating multipotent hematopoietic cells. These methods should help to further establish if there is a direct lineage relationship between neonatal repopulating cells and definitive HSCs.

To examine the lineage relationship between embryonic HSC and adult BM HSCs, very specific temporal and spatial molecular marking of HSCs is required. Thus, in this thesis research we attempted to mark HSCs generated in the AGM, and follow them through to the late fetal and adult stages to determine if they are the source of the entire adult hematopoietic hierarchy. Our strategy to perform such HSC tracing experiments was to genetically mark AGM HSCs at midgestation using a ligand (Tamoxifen/or 4OHT)-inducible Cre/loxP recombination system. To induce (by ligand administration) recombination in hematopoietic multipotent cells, we have used a transgenic line, which expresses Tamoxifen/4OHT-inducible Cre recombinase under control of regulatory elements of *Ly-6A* gene. This 14 kb genomic region of the *Ly-6A* was shown previously to drive expression of reporter gene (*GFP*) in all definitive HSCs both at embryonic as well as adult stages. We used 2 different lox reporter lines that upon Cre mediated recombination express either lacZ or EGFP. Summarizing our results, we detected the expression marker in hematopoietic cells in hematopoietic organs isolated from *in utero* Tamoxifen-treated double transgenic (Ly-6A CreERT: Rosa 26 lox lacZ) adult offspring. However, we were not able to precisely define if AGM HSCs contribute to adult HSC pool, since we observed only a very low number of hematopoietic cells expressing the recombination marker amidst a high non-specific staining background. Thus, to clarify whether definitive HSCs from AGM colonize the adult hematopoietic compartment, further improvements in recombination marking are needed, including increased recombination efficiency by applying a reliable lox reporter line and structural modifications of Cre ERT.

CHAPTER 7

Samenvatting

Samenvatting

In het embryo en de volwassen muis, vindt de vorming van hematopoietische cellen plaats in speciale weefsels die goed doorbloedt zijn. In het embryo zijn deze weefsels onder andere de dooierzak, de aorta-gonade-mesonephros (AGM) regio, de lever en de placenta. In de volwassen muis vindt hematopoiese voornamelijk plaats in het beenmerg. Ondanks verschillen in hematopoietische hierarchie tussen het embryonale en volwassen hematopoietische systeem, zijn de hematopoietische stamcellen (HSC) in beide systemen de meest potente cellen. HSC worden gekarakteriseerd door een hoge nucleus-cytoplasma volume ratio en door de expressie van specifieke moleculaire markers. HSC kunnen zich vermeerderen, maar kunnen ook differentiëren wat uiteindelijk resulteert in morfologisch en functioneel verschillende typen rijpe bloedcellen. Deze rijpe bloedcellen voeren de functies van het hematopoietische systeem uit, zoals het vervoer van zuurstof (door erythrocyten), de vernietiging van lichaamsvreemde cellen en/of organismen (door myeloide cellen) of het uitvoeren van een afweerreactie tegen ziekteverwekkers (door lymphoide cellen).

De keuze van HSC om een bepaalde richting op te differentiëren wordt niet alleen gereguleerd door dynamische veranderingen in de omgeving van HSC, maar ook door stamcel-intrinsieke factoren. Naar de moleculaire mechanismen die ten grondslag liggen aan de cel-intrinsieke regulatie van het ontstaan en de activiteit van HSC in het embryo wordt nog steeds intensief onderzoek gedaan. Een van de transcriptiefactoren die een belangrijke rol speelt in de ontwikkeling van HSC is GATA-2. Eerdere studies hadden al aangetoond dat de aanwezigheid van GATA-2 tijdens de embryonale ontwikkeling nodig is voor normale activiteit van HSC. In de studies die in dit proefschrift staan beschreven hebben we laten zien dat ook de hoeveelheid *GATA-2*, zoals een diploide dosis, van belang is voor de activiteit van HSC. Om het effect van de *GATA-2* genexpressie niveaus te bestuderen hebben we een aantal *in vivo* en *in vitro* experimenten uitgevoerd. Een verminderde expressie van GATA-2, zoals in *GATA-2*$^{+/-}$ embryos, had met name effect op de stamcellen die in de AGM regio onstaan. De AGM regio is het weefsel waar op embryonale dag 10.5 (E10.5) de eerste HSC worden gevormd. In de AGM regio was het ontstaan en de vermeerdering van *GATA-2*$^{+/-}$ HSC ernstig verstoord zowel in *in vivo* als in *in vitro* experimenten. In de dooierzak hebben we kleine veranderingen in activiteit van HSC waargenomen, terwijl in de lever in eerste instantie geen defect waarneembaar was. Echter, door het uitvoeren van opeenvolgende en competitieve transplantaties met HSC afkomstig van *GATA-2*$^{+/-}$ foetale lever of volwassen beenmerg, vonden we voor beide weefsels defecten in de HSC. Tevens hebben we laten zien dat een cytotoxische behandeling van *GATA-2*$^{+/-}$ volwassen muizen resulteerde in een proliferatie defect van HSC in het beenmerg. Met deze data hebben we aangetoond dat de mate van GATA-2 expressie een rol speelt in de regulatie van de groei en ontwikkeling van HSC gedurende de verschillende ontwikkelingsstadia.

Naast deze studies naar de moleculaire mechanismen die betrokken zijn bij de regulatie van HSC, is een belangrijk aspect van de vorming van HSC het achterhalen van de precieze cellulaire afkomst van deze cellen. Studies naar de embryonale ontwikkeling van de kip, hebben aangetoond dat endotheliale cellen de voorlopers zijn van hematopoietische cellen. Het bestaan van deze hemato-endotheliale cellen wordt verder onderbouwd met functionele *in vitro* en histologische studies. Echter, in de muis is nog geen direct verband tussen endotheliale cellen en HSC aangetoond. Sommige onderzoekers hebben voorgesteld dat multipotente hematopoietische precursors, die wel in neonatale, maar niet in volwassen muizen het hematopoietische systeem kunnen repopuleren, de directe voorlopercellen van HSC zijn. Dit soort multipotente precursor cellen zijn aanwezig in de dooierzak en in de P-Sp regio (voorloperweefsel van de AGM) nog voordat HSC die volwassen muizen kunnen repopuleren in de AGM worden gedetecteerd. Verder onderzoek naar deze cellen heeft aangetoond dat deze multipotente precursor cellen dezelfde markers op het celoppervlak hebben als HSC. De data die in dit proefschrift wordt gepresenteerd geeft vernieuwde inzichten in deze precursors die neonatale muizen kunnen repopuleren. In deze studies, waarin tevens werd gekeken naar de bruikbaarheid van een embryo *in vitro* systeem, is gebruik gemaakt van neonaten die een hematopoietisch defect hebben, de W41/W41 (c-kit mutant) muizen. De neonaten werden getransplanteerd met cellen afkomstig van genetisch gemarkeerde embryos die zich *in utero* of *in vitro* ontwikkeld hadden. De transplantatie van preE10 dooierzak en P-Sp cellen in deze W41/W41 neonaten resulteerde in langdurige en volledige hematopoietische reconstitutie. Hieruit kan geconcludeerd worden dat deze multipotente hematopoietische precursor cellen zich zowel *in utero* als in ons *in vitro* embryo-kweek systeem ontwikkelen. Aangezien de manipulatie van muizenembryos *in utero* vele technische moeilijkheden met zich meebrengt, is ons embryo-kweek systeem waarin deze multipotente precursor cellen zich ontwikkelen een aantrekkelijk alternatief. De embryo kweek geeft de mogelijkheid om verder uit te zoeken of er een directe relatie is tussen deze multipotente precursorcellen en de HSC die later tijdens de embryonale ontwikkeling ontstaan.

Om de relatie tussen HSC in het embryo en in het volwassen beenmerg verder te onderzoeken, is een markering van HSC op een specifiek tijdstip en plaats in het embryo noodzakelijk. Om te onderzoeken of de HSC die in de AGM regio ontstaan de bron vormt voor de HSC die in de volwassen muis zorg dragen voor het hematopoietische systeem, hebben wij in onze studies geprobeerd om HSC die in de AGM regio ontstaan te markeren en deze cellen te volgen tijdens de foetale ontwikkeling en in de volwassen muis. De strategie die we hiervoor gebruikt hebben was gebaseerd op het Tamoxifen-induceerbare Cre-lox recombinatie systeem. Om recombinatie in HSC te kunnen induceren, is het Tamoxifen-induceerbare Cre-recombinase gen geplaatst onder de regulatoire elementen van het Ly-6A gen. Een 14 kb gedeelte van het Ly-6A gen was al in andere studies gebruikt om een reporter gen (GFP) tot overexpressie te brengen in HSC tijdens de embryogenese en in de volwassen muis. We hebben 2 verschillende lox-reporter muizenlijnen gebruikt, die door Cre-

gemedieerde recombinatie of LacZ of GFP tot expressie zouden brengen. Samengevat hebben we reporter-gen expressie gevonden in hematopoietische cellen van Tamoxifen behandelde dubbel-transgene (Ly-6A CreERT: Rosa26-lox-lacZ) embryos die zich tot volwassen muizen hadden ontwikkeld. Helaas hebben we niet met zekerheid kunnen vaststellen of de HSC in de volwassen muizen afkomstig waren van AGM-HSC, aangezien er maar zeer kleine aantallen gemarkeerde hematopoietische cellen werden gedetecteerd en de achtergrondkleuring in deze experimenten zeer hoog was. We moeten daarom concluderen dat verdere verbeteringen van deze techniek voor het markeren van HSC door middel van recombinatie, waaronder veranderingen van de Cre ERT en verhoogde recombinatie efficiëntie, noodzakelijk zijn om te kunnen bestuderen of AGM-HSC een bijdrage leveren aan het hematopoietische systeem van volwassen muizen.

CHAPTER 8

Streszczenie

Streszczenie

Zarówno embrionalny jak i postnatalny rozwój systemu krwionośnego odbywa się w określonych, wysoce unaczynionych miejscach. W embrionie do takich miejsc zalicza się: woreczek żółtkowy, AGM (*aorta-gonad-mesonephros*), wątrobę i łożysko. W organizmie dorosłym hemopoeza ma miejsce głównie w (czerwonym) szpiku kostnym. Mimo że hierarchia układu krwionośnego różni się pomiędzy embrionem a organizmem dorosłym, najbardziej mulitpotencjalnym typem komórki dla obu systemów krwiotwórczych jest komórka macierzysta krwi. Taka komórka, charakteryzująca się wysokim stosunkiem jądrowo-cytoplazmatycznym oraz ekspresją specyficznych markerów molekularnych, jest zdolna do samoodnawiania i dawania początku tak morfologicznie jak i funkcjonalnie odmiennym komórkom krwi. Powstałe ze zróżnicowanych komórkek macierzystych dojrzałe komórki krwi pełnią liczne funkcje np.: transport istotnych gazów (erytrocyty), niszczenie obcych komórek lub organizmów (komórki mieloidalne) albo indukowanie odpowiedzi immunologicznej w obecności czynników chorobotwórczych (komórki limfoidalne).

Uważa się, że na decyzje dotyczące różnicowania komórek macierzystych krwi mają wpływ nie tylko dynamiczne zmiany mikrośrodowiska, ale także właściwe komórkom macierzystym czynniki wewnętrzne. Molekularne mechanizmy tej wewnętrznej regulacji powstawania komórek macierzystych krwi oraz regulacji ich aktywności pozostają nadal obiektem intensywnych badań. Jednym z czynników transkrypcyjnych istotnych dla rozwoju komórek macierzystych krwi jest gen *GATA-2*. Brak tego czynnika zaburza aktywność komórek macierzystych w embrionie, co zostało potwierdzone we wcześniejszych eksperymentach. Wyniki badań przedstawione w niniejszej pracy dowodzą, że poziom genu *GATA-2*, zwłaszcza jego diploidalna dawka, ma również wpływ na komórki macierzyste krwi. W celu określenia związku miedzy poziomem tego genu a rozwojem oraz aktywnością komórek macierzystych krwi, zarówno w dorosłych jak i embrionalnych kompartmentach krwiotwórczych, przeprowadziliśmy szereg testów *in vitro* oraz *in vivo*. Haploidalna dawka genu *GATA-2* wpłynęła głównie na krwiotwórcze komórki macierzyste w AGM regionie, tj. w części embrionu odpowiedzialnej za autonomiczną produkcję pierwszych ostatecznych (definitywnych) komórek macierzystych krwi (w embrionalym dniu E10.5). U osobników *GATA-2*$^{+/-}$ zaobserwowaliśmy również wysoce zredukowaną zdolność tworzenia i ekspansji komórek macierzystych krwi w AGM tak *in vitro* jak i *in vivo*. Aktywność komórek macierzystych krwi woreczka żółtkowego była tylko nieznacznie zaburzona, natomiast aktywność komórek macierzystych krwi z wątroby płodowej praktycznie nie uległa zmianie. W transplantacjach seryjnych oraz konkurencyjnych, zarówno w embrionalnych jak i dorosłych komórkach macierzystych krwi *GATA-2*$^{+/-}$ stwierdziliśmy wady jakościowe. Ponadto, zastosowanie cytotoksyków u dorosłych myszy *GATA-2*$^{+/-}$ obniżyło zdolności proliferacyjne komórek macierzystych szpiku. Uzyskane wyniki wyraźnie wskazują, że gen *GATA-2*, w sposób zależny od dawki,

uczestniczy we wzroście i namnażaniu się krwiotwórczych komórek macierzystych podczas mysiej ontogenezy.

Obok badań nad molekularnym programem wpisanym w regulację komórek macierzystych krwi, istotny aspekt badań nad tymi komórkami stanowi dokładne określenie ich prekursorów. Eksperymenty polegające na śledzeniu losu komórek macierzystych krwi przeprowadzone na kurzych embrionach pozwoliły zidentyfikować komórki endotelialne jako bezpośrednie prekursory komórek macierzystych krwi. Istnienie krwiotwórczych komórek endotelialnych zostało udowodnione zarówno w badaniach *in vitro* jak i histologicznych. U myszy bezpośredni związek pomiędzy naczyniowymi komórkami endotelialnymi a krwiotwórczymi komórkami macierzystymi nie został jednak jak dotąd wyjaśniony. Niektórzy badacze postulują, iż bezpośrednimi prekursorami komórek macierzystych krwi mogłyby być już częściowo zróżnicowane embrionalne, multipotencjalne komórki progenitorowe, które są w stanie odtworzyć układ krwionośny mysich noworodków, ale nie dorosłych biorców. Takie przeszczepione noworodkowi embrionalne macierzyste komórki krwi zostały wykryte w woreczku żółtkowym i P-Sp (z którego rozwija się AGM) przed pojawieniem się pierwszych komórek macierzystych krwi w AGM, które to komórki odtwarzają dorosły układ krwionośny. Fenotypowa i funkcjonalna analiza tych multipotencjalnych komórek wykazała, że ekspresjonują one na powierzchni błony komórkowej białka specyficzne dla komórek macierzystych krwi. Zamieszczone w niniejszej pracy dane eksperymentalne pozwalają dodatkowo scharakteryzować krwiotwórczą aktywność komórkek macierzystych odnawiających system krwiotwórczy noworodka. Metoda hodowli mysich embrionów w celu śledzenia losów komórek macierzystych krwi pozwoliła nam wykorzystać mysie noworodki mutanta W^{41}/W^{41} (c-kit mutanta) jako biorców w analizie krwiotwórczej aktywności wczesnych embrionalnych komórek macierzystych krwi. Przeszczep preE10 P-Sp i woreczka żółtkowego, wyizolowanych z genetycznie znaczonych embrionów mysich rozwiniętych *in utero* lub *in vitro*, do nie poddanych mieloablacji (zniszczeniu szpiku kostnego) noworodków W^{41}/W^{41}, doprowadził do długoterminowej, wieloliniowej repopulacji komórek pochodzących od dawcy. Multipotencjalne komórki macierzyste krwi, odtarzające krwiotwórczy system noworodka, rozwinęły się więc zarówno *in utero* jak i *in vitro*. Ze względu na duże trudności techniczne w manipulacji mysimi embrionami *in utero*, hodowla mysich embrionów *in vitro* stanowi bardzo atrakcyjną metodę pozwalającą na właściwy rozwój multipotencjalnych krwiotwórczych komórek macierzystych krwi zdolnych do repopulacji systemu krwionośnego noworodka. Obie wyżej wspomniane metody powinny pomóc odpowiedzieć na pytanie czy istnieje bezpośredni związek pomiędzy komórkami odtwarzającymi system krwiotwórczy noworodka a ostatecznymi komórkami macierzystymi krwi.

W celu dalszego zbadania relacji pomiędzy embrionalnymi komórkami macierzystymi krwi a dorosłymi szpikowymi komórkami macierzystymi krwi niezbędne jest bardzo specyficzne, czasowo-przesterzenne, molekularne oznakowanie krwiotwórczych komórek macierzystych powstających w AGM. Zatem, aby określić

czy komórki macierzyste wyprodukowane w AGM stanowią źródło komórek krwiotwórczych u osobników dorosłych podjęliśmy w niniejszej pracy próbę oznakowania i śledzenia losu tych komórek podczas późnych embrionalnych i dorosłych stadiów rozwojowych. W naszych eksperymentach embrionalne komórki macierzyste krwi z AGM zostały genetycznie oznakowane w trakcie ciąży poprzez zastosowanie indukowanego ligandem (Tamoxifen/lub 4OHT) systemu rekombinacyjnego Cre/loxP. W celu zainicjowania recombinacji w multipotencjalnych komórkach macierzystych, użyliśmy transgenicznej mysiej linii, która ekspresjonuje rekombinazę Cre indukowaną przez Tamoxifen/4OHT pod kontrolą elementów regulatorowych genu *Ly-6A*. Wcześniejsze badania wykazały, że region genomu o masie 14 kb zawierający gen *Ly-6A* indukował ekspresję genu reporterowego (GFP) we wszystkich ostatecznych komórkach macierzystych w trakcie rozwoju tak embrionalnego jak i dorosłego. Do wykrycia rekombinacji użyliśmy dwóch różnych mysich linii transgenicznych z reporterem lox, które w wyniku rekombinacji przeprowadzonej przez Cre, ekspresjonują β-galaktozydazę lub GFP.

Podsumowując, uzyskane przez nas wyniki pozwoliły nam wykryć ekspresję markera rekombinacji w komórkach macierzystych krwi w narządach krwiotwórczych pochodzących z podwójnie transgenicznego (Ly-6A CreERT: Rosa26 lox lacZ) dorosłego potomstwa, które zostało uprzednio (*in utero)* poddane iniekcjom Tamoxifenem. Bardzo mała liczba krwiotwórczych komórek macierzystych ekspresjonujących marker rekombinacyjny przy wysokim niespecyficznym tle nie pozwoliła nam jednak dokładnie ustalić czy komórki macierzyste z AGM wchodzą w skład puli dorosłych krwiotwórczych komórek macierzystych. Aby odpowiedzieć na pytanie czy ostateczne komórki macierzyste z AGM kolonizują kompartment krwiotwórczy dorosłego osobnika potrzebne są dalsze ulepszenia w rekombinacyjnym znakowaniu, w tym podniesienie wydajności rekombinacji poprzez zastosowanie niezawodnych transgenicznych linii myszy z reporterem lox oraz modyfikacji strukturalnych białka fuzyjnego CreERT.

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