The Role of the Embryonic Microenvironment in Hematopoietic Cell Development

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De rol van de embryonale micro-omgeving op de ontwikkeling van hematopoietische cellen

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Voor jou

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Chapter 1

General Introduction

1.1 The hematopoietic system

Over the last decades much progress in our understanding of mechanisms involved in the regulation of the hematopoietic system has been made. These studies mainly focus on the so-called 'mother of all blood cells', namely the hematopoietic stem cell (HSC). Blood contains a wide range of cell types, each providing a unique function. There is a lymphoid compartment (T- and B-lymphocytes) comprising the adaptive immune system, and a myeloid compartment necessary for oxygen transport, blood clotting and immune response (erythrocytes, macrophages, dendritic cells, platelets and granulocytes). Most mature blood cells display a limited life span and therefore a continuous replacement of these cells is needed during life. The generation of all the cell types starts at the level of the HSC. Through a sequential series of proliferation and differentiation events, a HSC gives rise to progenitor cells with increasingly restricted lineage potential, and it is these cells that eventually produce terminally differentiated mature blood cells.

There is a critical balance between preserving HSCs in an undifferentiated state and generating mature blood cells. This is a tightly regulated process. A disturbed balance can lead to various pathological conditions including leukaemia or anaemia. HSCs are maintained through the supportive properties of the microenvironment. This microenvironment changes during the development of an organism. Although HSCs are self-renewing throughout adult life, they take their origins during embryonic development. Thus a major question in the field is how is the adult hematopoietic system generated during embryonic development and at which embryonic sites?

1.1.1 The hematopoietic system during embryonic development

The establishment of the hematopoietic system in the mouse embryo begins in the blood islands of the yolk sac and leads to the appearance of primitive erythroid cells at embryonic day 7.5 (E7.5) [118] (Figure 1). Within the blood islands, the hematopoietic and endothelial cells emerge simultaneously and in close association with each other [143]. The cells at the periphery of the blood islands develop into endothelial cells whereas the inner cells form primitive erythroid cells [70]. Since both types of cells are arising from the same region within the yolk sac, it was suggested that they emerge from a common progenitor called the haemangioblast. Indications for the existence of the haemangioblast were given by the presence of a clonal precursor *in vitro* and that some gene mutations affect both cell populations [20,76]. An elegant gene targeting study by Shalaby and co-workers [162] showed that in the absence of endothelial-specific vascular endothelial growth factor receptor 2 (VEGFR2) both endothelial and hematopoietic cells are absent in the developing embryo. Formation of the yolk sac blood island was completely absent at E7.5 in these deficient mice. The importance of VEGFR2 for the survival of the haemangioblast was shown by Eichmann and co-workers [42]. They isolated VEGFR2 expressing cells from the mesoderm of chicken embryos. These cells were able to give rise to hematopoietic and endothelial cells *in vitro*. Using mouse embryonic stem cells (ESCs) a haemangioblast-like cell has been identified and called the blast-colony forming-cell (BL-CFC) [20,76]. This *in vitro* equivalent of the haemangioblast is able to give rise to both hematopoietic and endothelial cells upon stimulation by

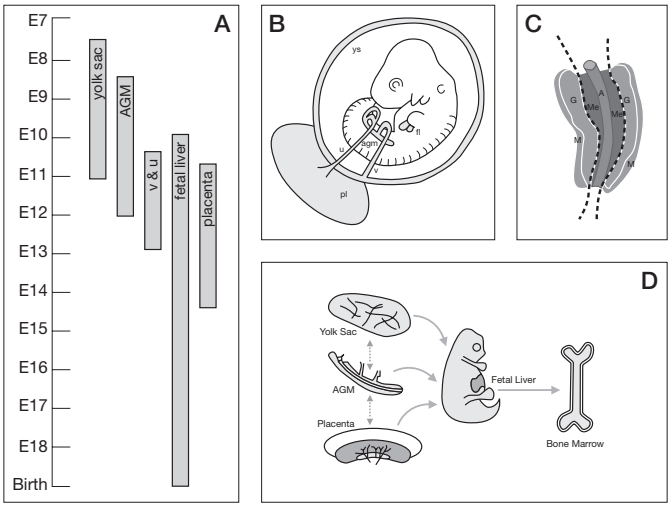

Figure 1. Schematic overview of the hematopoietic system during mouse embryonic development
(A) The distribution of hematopoietic cells during mouse development. Primitive erythroid cells appear in the yolk sac at E7.5 followed by CFU-S detected in the PAS/AGM region. At late E9 the first hematopoietic cells arrive in the fetal liver, which from there on will remain the main hematopoietic site during embryonic development until birth. The first long-term adult repopulating HSCs appear in the AGM region at E10.5, but they are also found in the vitelline and umbilical (v&u) arteries. Furthermore, at E12 the placenta harbours HSCs. Around birth the hematopoietic cells are thought to go and colonize the bone marrow (not shown). (Adapted from the Phd thesis of A. Oziemlak 2005). **(B)** Schematic representation of a mid-gestation mouse embryo. Indicated in grey are the hematopoietic sites during mouse embryonic development. Yolk sac (YS) and placenta (PL) are the extra-embryonic sites and the aorta-gonads-mesonephros (AGM) region, fetal liver (FL) are the intra-embryonic sites. The vitelline (V) and umbilical (U) arteries connect the intra- and extra-embryonic sites [41]. **(C)** Schematic representation of the AGM region. The dorsal aorta (A) is located in the midline surrounded by the mesenchyme (Me). Juxtaposed laterally are the gonads (G) and the mesonephros (M) that together form the urogenital ridges [28]. **(D)** Current model for HSCs generation and migration during embryonic development. HSCs are generated independently in the AGM region. The yolk sac and the vitelline and umbilical arteries and placenta also harbour HSCs. Emerging data suggest that via circulation HSCs can be cross-seeded between the tissues (dashed lines). All of these tissues may contribute to the pool of HSCs that colonize the fetal liver (solid lines) where they may be expanded. Just before birth the HSCs and progenitor pool will migrate to the bone marrow, where they are maintained during adult life [140].

VEGF (the ligand of VEGFR2) and stem cell factor (SCF). *In vivo* the presence of the BL-CFC expressing VEGFR2 in the posterior region of the primitive streak (E7-7.5) in mouse embryos provided additional evidence for a common progenitor [62].

For several decades it was thought that the hematopoietic cells generated in the yolk sac blood islands established the adult system via colonization of the fetal liver and subsequently the bone marrow. However, studies performed on avian and amphibian embryos challenged this idea [33,188]. A mesodermally derived region surrounding the aorta was found to harbour intra-embryonic hematopoietic activities. This suggested that the mammalian system might harbour hematopoietic activity in a similar

intra-embryonic site. In the 1990s several studies showed that in the mid-gestation mouse embryo such sites exist [54,104]. Godin and co-workers [54] identified hematopoietic activity in the para-aortic splanchnopleura (PAS) region at E9 with the potential to produce a subset of B-lymphoid cells. This PAS region is the equivalent of the mesodermal derived region determined in the avian and amphibian embryos. At the same time Medvinsky and co-workers [104] identified the presence of the multipotent CFU-S (for explanation see chapter 1.1.3) within the PAS-region at E9. Closer analysis showed that after the emergence of CFU-S at E9 their numbers increase until late E10 within this region. In the same study they showed the presence of CFU-S in the E9 yolk sac. Strikingly, the number of CFU-S present in the yolk sac is less than in the PAS region. Further in development (after E10) the CFU-S numbers in yolk sac and PAS region decrease rapidly while at the same time an increase is observed in the fetal liver. These observations lead to the assumption that cells from the PAS region and yolk sac colonize the fetal liver.

Later in development the PAS will give rise to the aorta, gonads and mesonephros (AGM) region (Figure 1). Beginning at mid-E10, HSC activity was found within the AGM region [120]. HSC activity was also found in yolk sac and fetal liver at E11. Medvinsky and co-workers [103] used an organ culture system to show that the AGM region (at E10.5) is the first intra-embryonic site able to generate HSCs. In this organ culture system, yolk sac and fetal liver explants failed to generate HSCs at this stage. Closer investigation of the AGM region by de Bruijn and co-workers [28] showed that the first HSCs arise in the aorta subregion of the AGM (Figure 1) at E11. Also the other large vessels of the embryo, the vitelline and umbilical arteries harbour HSCs at this time in development and thus are suggested to be important in HSC emergence. Later in ontogeny the urogenital subregion contains HSCs. However, it is still undetermined whether this subregion is colonized or generates HSCs by itself. Recently, Kumaravelu and co-workers [80] used a quantitative approach to analyse the development of HSCs in the mouse embryo. Their data indicated that at E12 the yolk sac is able to generate HSCs using an organ culture system. However, E12 fetal liver failed to generate HSCs in this experimental set up. Strikingly, at E11 and E12, HSCs accumulate in the fetal liver *in vivo*. Since the fetal liver failed to generate HSCs *in vitro* at these time points, it was suggested that the accumulation of HSCs in the fetal liver *in vivo* could be the result of colonization of HSCs from the AGM region and the yolk sac through the circulation.

To determine more precisely where HSCs are generated within the embryo, de Bruijn and co-workers used a transgenic approach [25]. They used a transgenic mouse model expressing a GFP marker under the control of the Sca-1 (Ly-6A) locus. Sca-1 is a well-known HSC marker (Table 1). Analysis of transgenic embryos showed that Ly6A-GFP was expressed in all HSCs in the AGM region. More precisely, immunohistological analysis revealed transgene (GFP) expressing cells were located at the endothelial cell layer, lining the wall of the dorsal aorta and the vitelline and umbilical arteries. Also cells budding from the aortic wall, referred to as hematopoietic clusters, expressed GFP [140]. Considering that the functional HSCs in the AGM region all express the transgene they concluded that the first HSCs are generated in the endothelial cell layer lining the dorsal aorta and from there they will bud into the aorta lumen forming clusters of HSCs. Furthermore, expression of the transgene was observed in E11 fetal liver and E11 yolk sac hematopoietic cells and all HSCs in the E12 placenta [140]. Thus the

Ly6A-GFP transgene is expressed in all HSCs at distinct hematopoietic sites in both the embryo and adult.

The presence of hematopoietic cell clusters in the aorta and other major arteries during development [34] suggest that within the intra-embryonic site there is a developmental relationship between endothelial and hematopoietic lineages. As indicated before, these clusters harbour HSCs [25]. Suggestions were raised that there is a haemangioblast present in the mesenchyme of the AGM region giving rise to both cell populations [70]. Progeny of the haemangioblast cells are thought to migrate up to the aorta where they will reside as endothelial cells forming the aorta wall or they migrate through the vessel wall as hematopoietic cells into the circulation [70]. An alternative hypothesis was made that the hematopoietic cells are generated through endothelium intermediate cell types called haemogenic endothelium. Recent studies suggested that the presence of a haemogenic endothelium is more likely. DiI-AcLDL labelling of the endothelial tree in chicken embryos, prior to the presence of hematopoietic clusters, revealed that emerging hematopoietic clusters were labelled, indicating the presence of endothelium-derived progenitors seeding the clusters [69]. Similar results were obtained within the mouse embryo [175].

From E11 onwards HSCs are detected in the AGM region, yolk-sac, circulation and the fetal liver [80,103,120]. Within the yolk sac and AGM the numbers of HSCs present are declining rapidly over time (until E13), while the numbers in the fetal liver are increasing. Recently, Gekas et al. and Ottersbach et al. determined the presence of HSCs in the placenta. From E11 onwards there is an increase in the number of HSCs reaching a maximum at E13 and thereafter declining [52] (Figure 1). Importantly, the appearance of HSCs in the placenta is simultaneous to the appearance in the AGM and yolk sac (E11), but before the presence of HSCs in the fetal liver. Another important detail is that the HSCs in the placenta appear before any HSC can be detected in the circulation. Furthermore, they express HSC-markers and transcription factors suggesting that the placenta is involved in the generation and/or maturation of HSCs. From E15 until birth the fetal liver remains the main hematopoietic site in the embryo, but the developing thymus, spleen and blood also harbour hematopoietic cells. Just before birth the HSCs and progenitors present in the liver are thought to migrate to the bone marrow, where they create the main hematopoietic site during adult life.

Overall, the development of the hematopoietic system during gestation is a highly dynamic process. HSCs are autonomously generated in the AGM region, but are likely to be generated also in the vitelline and umbilical arteries, yolk sac and placenta. All tissues are thought to contribute to the colonisation of the fetal liver (Figure 1). Apparently these tissues provide an ideal hematopoietic microenvironment for generation, expansion and/or maintenance of hematopoietic progenitors and HSCs.

1.1.2 The hematopoietic system in adult life

The adult hematopoietic system is comprised of a hierarchy of cells with the pluripotent HSC at the foundation [153] (Figure 2). The pluripotent HSCs give rise to lineage-restricted progeny that differentiate into fully matured hematopoietic cells. During this differentiation process two compartments are generated: the lymphoid and myeloid compartment. The lymphoid compartment will give rise to T

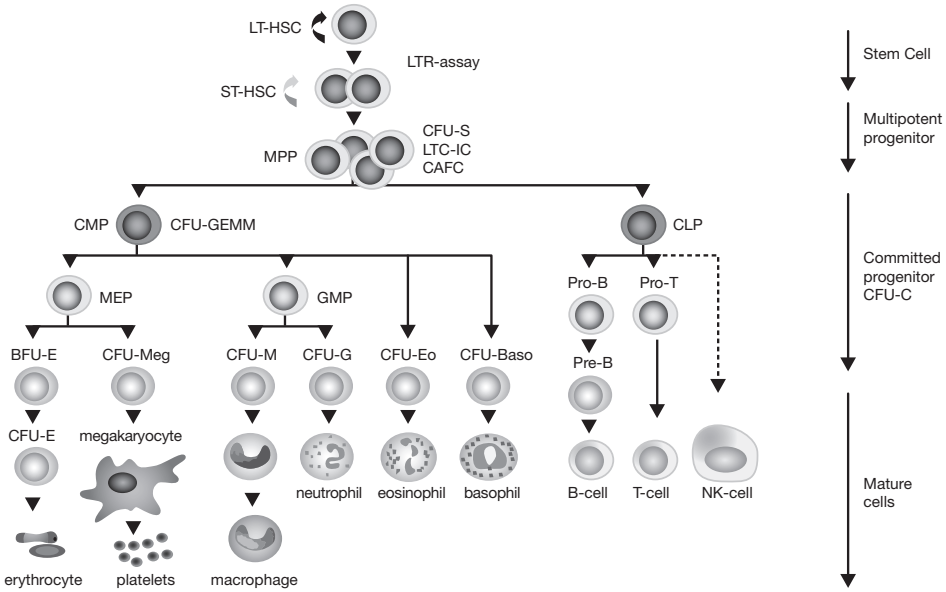

Figure 2. Scheme of the hematopoietic hierarchy in adults
Long-term HSCs (LT-HSC) are at the origin of the adult hematopoietic system giving rise to short-term HSCs (ST-HSC). Both types of HSCs display self-renewal capacity. HSCs are assayed through *in vivo* multilineage repopulation. LT-HSCs give rise to life-long replacement of the complete hematopoietic system, whereas ST-HSCs function only for 4-6 weeks after transplantation due to less self-renewal capacity. ST-HSCs give rise to multipotent progenitors (MPP). Analysis of MPPs is by an *in vivo* CFU-S (colony forming unit-spleen) assay or by *in vitro* LTC-IC (long-term culture-initiating cell) or CAFC (cobblestone area forming cell) assays. These MPPs will differentiate into either lymphoid (CLP) or myeloid (CMP) committed progenitors, which in turn will give rise to the lineage restricted mature blood cells. Indicated in this scheme are also *in vitro* assayed progenitors. These different CFU-Cs (colony-forming units-culture) can be analysed in methylcellulose cultures. Indicated are: CFU-GEMM (granulocyte, erythrocyte, macrophage, megakaryocyte), BFU-E and CFU-E (erythrocyte), CFU-Meg (megakaryocyte), CFU-M (macrophage) and CFU-G (granulocyte), CFU-Eo (eosinophil) and CFU-Baso (basophil). (Adapted from: Lund Stem Cell Centre; http://www.stemcellcenter.se)

and B-lymphocytes and natural killer (NK) cells. The myeloid compartment gives rise to eosinophils, basophils, neutrophils, macrophages, platelets and erythrocytes [153]. However, recently Adolfsson and co-workers [2] proposed a separate model in which HSCs are initially primed to undergo myeloid commitment. This divides the more committed cells into two populations. First, the myeloid population (CMP) gives rise to megakaryocytes and erythrocytes, but also maintains the granulocyte and macrophage potential. The second population harbours a lymphoid-primed multipotent progenitor (LMPP), which will be committed to the lymphoid fate by activation of lymphoid genes. This population also has granulocyte and macrophage potential, but will give rise to the lymphoid compartment (T- and B-lymphocytes). Furthermore, the LMPP completely lacks any erythroid or megakaryocytic potential. The strict separation between the lymphoid and myeloid compartment suggested by Reya and co-workers [153] is still widely accepted as a model. Recent evidence supporting this hierarchy model

was provided by the group of Forsberg [48]. The LMPP determined by Adsolfsson was suggested to represent a more committed subpopulation of the multipotent progenitors (MPPs). However, we have to keep in mind that the current proposed models should only be seen as a simplification of complex biological processes.

HSCs and hematopoietic progenitors reside within the bone marrow during adult life. The spleen, thymus, blood vessels and lymph nodes also harbour hematopoietic cells. The thymus and lymph nodes are exclusively lymphoid tissues while the bone marrow, spleen and blood vessels harbour cells of myeloid, erythroid and lymphoid lineages. Maturation of T- lymphocytes occurs in the thymus. Mature T-lymphocytes are found not only in the thymus but also in the spleen and lymph nodes. These lymphocytes are involved in regulation of the immune response by producing cytokines, destroying virally infected cells and tumour cells and plays a role in the maintenance of the immunological tolerance. B-lymphocytes mature in the bone marrow and are thereafter found in the spleen and lymph nodes. They produce antibodies, which assist in the destruction of antigens by binding to them. Hereby, these antigens are easier targets for phagocytosis. Another cell type involved in the bodies defence is the natural killer (NK) cell, which matures in the bone marrow. Mature NK-cells are later also found in the lymph nodes and blood vessels where they attack cells expressing foreign proteins. Maturation of erythrocytes occurs in the bone marrow after which they are released in the blood stream. They are involved in oxygen and CO_2 transport from and to the lungs. Old or damaged erythrocytes are destroyed in the spleen. Another type of cell in the hematopoietic system is the platelet, which is a cell fragment arising from megakaryocytes in the bone marrow. Platelets are able to initiate blood clotting in damaged blood vessels. Macrophages arise from the monocytes within the bone marrow and they phagocytose and digest invading microorganisms and foreign bodies. Damaged and senescent cells from the body are also removed by the macrophages. The eosinophils, basophils and neutrophils together are named granulocytes, which mature in the bone marrow. They are involved in defence response to bacteria and parasites and modulate allergic inflammatory responses.

1.1.3 Characterisation of HSCs and progenitors *in vivo* and *in vitro*

HSCs comprise a very rare population in the adult bone marrow. Only 1 in every 100.000 bone marrow cells is an HSC. Since these HSCs are at the top of the adult hematopoietic hierarchy, they are characterized by their ability to generate all the different hematopoietic lineages and they are able to self-renew.

Currently, the gold standard assay to detect HSCs is by *in vivo* transplantation and long-term repopulation (LTR) assay (Figure 2). In this assay, marked donor cells are injected into irradiated recipients and chimerism of the hematopoietic system is analysed for donor cells several months after injection (LTR-HSCs). Performing *in vivo* secondary transplantations can assess the self-renewal capacity of the donor HSCs. The donor cell-markers most frequently used are transgenes, like *LacZ* or *GFP*. Furthermore, the Y-chromosomal marker YMT (male marker) or mouse strain specific alleles like Ly5.1/5.2 are used. Via limiting dilution of the cells injected into recipients a quantitative analysis of the HSCs can be made. After analysing the donor cell contribution, the frequency of HSCs within

the donor cell population can be calculated. Using the competitive long-term repopulation assay the quality of the HSCs can be determined. In this assay donor cells are co-injected with competitor cells. After injection these populations will compete based on their homing and/or proliferative properties. The most potent HSCs will compete effectively and lead to engraftment of the recipients. The major advantage of the LTR assay is that true HSC activity is addressed within the natural environment. When HSCs lack homing capacity or are defective in proliferation and differentiation capacities they will not give rise to donor cell contribution in the recipient blood system.

Using the *in vivo* transplantation method short term repopulating (STR) HSCs can also be analysed. STR-HSCs engraft primary recipients by homing to the bone marrow and spleen and rapidly proliferate. This is followed by multilineage differentiation within the first weeks after injection. The main difference between LTR-HSCs and STR-HSCs is that the latter are limited in their self-renewal capacity. These cells are not able to serially engraft. Another *in vivo* method to analyse multipotent hematopoietic progenitors is the colony forming unit-spleen (CFU-S) assay. In this assay a population of test cells is injected into lethally irradiated recipients and after 8-12 days macroscopic nodules on the spleen can be quantified. The colonies that are quantified at day 8 after injection are more committed progenitors than are the colonies at day 12.

The use of *in vitro* assays circumvents the problems in the homing ability that may affect some HSCs and progenitors *in vivo*. However, as yet no *in vitro* assay can completely address the full function of HSCs. *In vitro* assays determine multipotency and/or lineage commitment of hematopoietic progenitors and HSCs. Two types of *in vitro* assay were developed for HSCs and multipotent progenitors; the LTC-IC assay (long-term culture-initiating cell) and the CAFC assay (cobblestone-area-forming-cell). Both assays use a co-culture system where the hematopoietic cells are cultured on top of a supportive stromal cell layer. The hematopoietic cells will form colonies in this appropriate environment. In the LTC-IC assay [110,177] hematopoietic cells are cultured up to 5-8 weeks, the co-cultures are harvested and analysed for their hematopoietic progenitor content using a CFU-C assay (explained below). The persistent growth of progenitors up to 8 weeks in the LTC-IC cultures suggests that HSCs are present. The CAFC assay [146,147] uses a similar co-culture system and assesses the presence of HSCs, multipotent and committed progenitors simultaneously. The test population of cells is seeded in a serial dilution manner and scored for the presence of cobblestone areas. A cobblestone area appears as a colony underneath the stromal cell layer and contains primitive progenitor cells. The cultures are scored between 7 and 35 days after plating. An advantage of the CAFC assay is that from the same sample, different stages of differentiated progenitors can be analysed. The cobblestones that appear at the beginning of the cultures (day 7, 10 and 21) are committed hematopoietic progenitors, whereas the cobblestones at day 28 and 35 are primitive hematopoietic progenitors. While correlations of the results of the same test population can be made between *in vivo* LTR assays and LTC-IC and CAFC assays, once plated *in vitro* HSCs no longer are functional *in vivo* repopulating cells.

The first *in vitro* assay to measure hematopoietic progenitor potential was the CFU-C assay (colony-forming unit-culture) [106]. CFU-C *in vitro* assays directly addresses the progenitor content of the test population by seeding them into semisolid medium supplemented with a well-defined cytokine and

growth factor cocktail. The different cytokines and growth factors used in these methylcellulose cultures allow the differentiation of subsets of hematopoietic progenitors. The morphological appearance of differentiated cells in the colonies at day 3, 7 and 14 after initiation (Figure 2, different types of CFU-C) allows determination of lineage potential of the different types of progenitors.

More precise studies of HSCs can be achieved by isolating and enriching this rare cell type. Although exclusive markers of HSCs have not been determined enrichment of HSCs has been achieved by using multiple markers and/or combining this with other techniques. One of the first techniques to enrich for HSCs was fractionation by a density gradient [192]. Using this method cells can be separated by differences in their densities using centrifugation. Media such as Ficoll, Percole or Hypaque, create different densities when laid on top of each other (the densest layer at the bottom and the cell suspension on top). The so-called "low-density" cell fraction contains the HSC population. The density gradient allows an HSC enrichment of 2-5 fold. Another technique takes advantage of the differential expression of proteins on the cell surface of hematopoietic cells. Fluorescent labelled antibodies against these surface proteins were developed and used in fluorescence-activated cell sorting (FACS). Subsequently, cells can be separated by the specific binding of antibodies (with the fluorescent dyes) against cell surface markers. This technique also allowed the separation of numerous differentiated hematopoietic cell populations, progenitors and HSCs, from a heterogeneous cell population, e.g. bone marrow. Some of the surface proteins (cell markers) expressed on HSCs are summarised in Table 1. However, most of the cell markers are expressed on more than one cell type. Therefore, combining specific markers during cell sorting are required and improves the enrichment of HSCs [158,171]. It is also possible to combine this technique with density gradient fractionation. Some examples of these combinations are discussed below.

Lineage-committed and/or mature hematopoietic cells can be separated from the more primitive hematopoietic cells by using a combination of markers like CD4/8 (T-cell), CD3 (T-cell), B220 (B-cell), Gr-1 (granulocyte) and TER119 (erythroid). All these markers are expressed on lineage-committed and mature cells, but not on the primitive hematopoietic cells. The use of combinations of antibodies specific for these lineage markers separates the HSC and progenitor populations (Lin^-) from the lineage-committed cells (Lin^+). Therefore this antibody cocktail is named Lineage Cocktail (Lin-cocktail). The Lin-cocktail is often used as a pre-enrichment step for HSCs of the bone marrow, achieving up to ~10-fold enriched HSC population [169]. The Lin^- population from bone marrow can be further enriched for HSCs by selecting the cells according to cell surface markers summarised in Table 1. A commonly used combination is Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ $CD34^{low}$ [139]. Another interesting procedure used for HSC enrichment is to first collect "low-density" cells from bone marrow and then stain them with antibodies specific for CD31 (PECAM/ER-MP12), Ly-6C (ER-MP20) and c-kit (CD117). Cell sorting of $CD31^{med}$ $Ly\text{-}6C^-$ $c\text{-}kit^+$ yields a highly enriched HSC population. Limiting dilution LTR assays performed on this particular population showed that 1 in every 300 cells is a LTR- HSC [132].

Enrichment of LTR-HSCs from embryonic tissues has been performed using cell-sorting techniques. Sanchez and co-workers [158] determined that the first LTR- HSCs in the AGM region and fetal liver all express the c-kit marker (Table 1). CD34 was already known to be expressed on LTR-HSCs

[158] so combining these two markers was an obvious step in enriching LTR-HSCs. Hence, Sanchez and co-workers demonstrated that LTR-HSC activity was present within the $CD34^+$ $c\text{-}kit^+$ (double positive) population of the AGM region and fetal liver (E11). These markers are now routinely used for the enrichment of LTR-HSC from E11 tissues including the aorta, vitelline and umbilical arteries, yolk sac and placenta.

Table 1. Hematopoietic (Stem) Cell Markers
(Updated and adapted from PhD thesis of C. Orelio 2003)

Marker	Alternative name(s)	Tissue and/or cell expression	Remarks	References
Lin-cocktail	CD4/8 or CD3 B220/CD45R TER119/Ly-76 Ly-6C and Ly-6G/Gr-1	T-lymphocytes B-lymphocytes erythroid cells myeloid cells (granulocytes and monocytes)	Is used to deplete lineage-committed hematopoietic cells.	169
Sca-1	Ly-6A/E	HSCs and progenitors lymphoid and myeloid cells vascular endothelium and stromal cells	Used in combination with Lin-cocktail and c-kit to enrich for HSC.	98 99 109
ER-MP20	Ly-6C	monocytes and macrophages endothelial cells neutrophils and T-cells bone marrow	antigen involved in differentiation of monocytes, macrophages and endothelial cells Regulated by interferon gamma lymphocyte development and maturation	29 26
CD31	PECAM ER-MP12	endothelial cells and fibroblasts HSCs and progenitors lymphoid and myeloid cells platelets and osteoclasts	plays a role in the removal of neutrophils Expressed in vascular tumours	67 82
CD34		HSCs and progenitors early myeloid and erythroid cells vascular endothelium	functions as adhesion factor mediates attachment between HSCs and niche	209 101 160
CD117	c-kit SCF-receptor	HSCs and progenitors vascular endothelium tissue mast cells, embryonic brain melanocytes, reproductive system	membrane receptor for stem cell factor (SCF)	5 13
CD41	Integrin alpha 2b	megakaryocytes and platelets HSCs in yolk sac and AGM	forms a receptor complex with CD61 functions in cell adhesion	108 111
CD105	endoglin	HSCs and endothelial cells vascular endothelium stromal cells and bone marrow monocytes and macrophages	Regulatory component of the TGF-beta complex	19 18
CD45	common leukocyte antigen	all hematopoietic cells HSCs	not expressed on erythrocytes	82 9
Flk-1	VEGF-R2	HSCs (adult and embryo) vascular endothelium embryonic stem cells (ES cells)	involved in growth of endothelial cells	162 201 203
FGF-R1	basic-FGF-R	HSCs (LSK cells) skin, hepatocytes	chromosomal translocations involved in leukaemia	30

1.2 HSCs and the balance between self-renewal and differentiation

The balance between HSC self-renewal, differentiation and proliferation is thought to depend on intrinsic and extrinsic factors. Intrinsic factors, like transcription factors, are involved via activation or inhibition of target gene expression within the HSCs. Extrinsic factors represents the influences from outside on the HSCs. This could be via growth factors and cytokines or via signals supplied by a so-called hematopoietic-inductive microenvironment or stem cell niche [24,161]. The intrinsic and extrinsic factors can influence the way whereby HSCs produce progeny, providing a manner to balance between self-renewal and differentiation. Increasing data [60,174,180] showed that HSCs are able to produce non-identical daughter cells, which could occur either via pre- or post-cell division events [196]. The pre-cell division asymmetry (Figure 3A) is achieved during mitosis, where one daughter cell receives all the determinants for maintaining stem cell features by the regulation of the location of the cleavage plane [196]. The other daughter cell harbouring determinants signalling differentiation will differentiate.

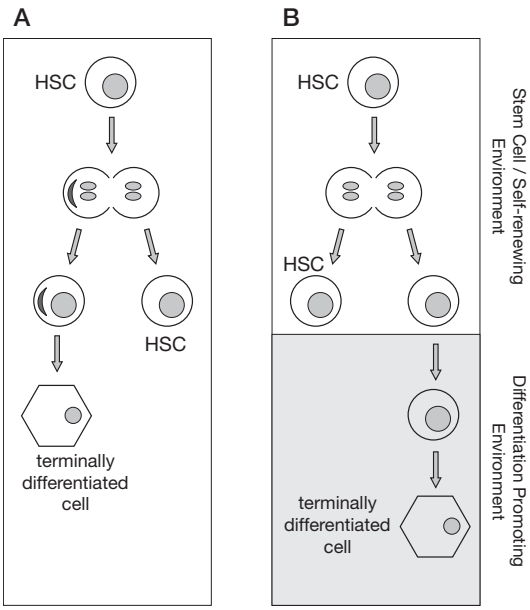

Figure 3. Two mechanisms producing non-identical daughter cells
(A) Pre-cell division mechanism. During asymmetric pre-cell division the cell-fate determinants are distributed to one of the future daughter cells during mitosis. These determinants signal differentiation in the daughter cell harbouring them, leading to terminally differentiated hematopoietic cells. The other daughter cell lacks these determinants and retains her stem ness. **(B)** Post-cell division mechanism. Asymmetry is obtained by the relocation of daughter cells in a differentiation-promoting environment. First the HSC will divide into two identical HSC-daughter cells. One of them will remain in the self-renewing environment. The other daughter will locate to the differentiation-promoting environment, where differentiation is initiated leading to a terminally differentiated hematopoietic cell [196].

The post-cell division asymmetry (Figure 3B) involves the exposure of the daughter cells to different environments. Hereby, after symmetric cell division one daughter cell remains in the environment conserving the stem cell features. A different environment that produces signals favouring differentiation surrounds the second daughter cell. So far it is thought that both mechanisms are important [196].

Another aspect influencing the HSC population in their ability to maintain the self-renewal and differentiation balance remains in the heterogeneity of this population. It was predicted that the complete adult HSC pool is a heterogeneous population. Some HSCs will divide while others are quiescent. Previously, Fleming and co-workers [47] showed that the most potent HSCs are in the G_0/G_1 phase (quiescent) and comprise up to 90% of the total HSC population within bone marrow. These quiescent bone marrow HSCs are mainly LTR-HSCs providing long-term multilineage engraftment of transplanted recipients [170]. Also, these quiescent HSCs serve as a spare pool, which can be activated in response to injury or stress [127]. The identification of the importance of cell-cycle status of HSCs is mainly performed in the adult hematopoietic system. Within the embryo the situation is thought to be very different. It was proposed that during embryonic development an enormous proliferation of the HSC pool occurs. The embryonic HSCs are therefore thought not to be quiescent but dividing [47] yet still retaining their stem cell properties. An example is the fetal liver HSC. It undergoes massive proliferation and is still able to provide long-term multilineage engraftment of transplanted recipients [189].

Recent studies implicated an important role for the stem cell niche in maintaining the balance between self-renewal and differentiation of HSCs and progenitors. The following paragraph will introduce the stem cell niche, growth factors, cytokines and transcription factors in relation to the influences upon HSCs and progenitors in more detail.

1.3 The stem cell niche

In the 1970s Schofield and co-workers proposed that HSCs are associated with other cell types in the bone marrow. These surrounding cells were thought to play a role in the way HSCs maintain the balance between self-renewal and differentiation. However, it took until the beginning of this century before this concept was supported by data on molecules and cell types involved in this microenvironment, which has become known as the hematopoietic niche [50,124,127,172].

The current view on the hematopoietic niche is that there are different types of niches performing different supportive functions for hematopoietic progenitors and stem cells. The first niche contains mainly quiescent HSCs and is named the quiescent-storage niche [127] (Figure 4). The cells comprising this niche provide an environment suppressing proliferation and differentiation of HSCs. However, it was suggested that under stress conditions the niche-cells signal the quiescent HSCs to become proliferative. Thereafter, they will start proliferating and differentiating to generate mature blood cells overcoming the stress situation. After re-establishment of the steady-state situation, they will become quiescent again. Another type of niche is called the self-renewing niche [122] (Figure 4). Its main func-

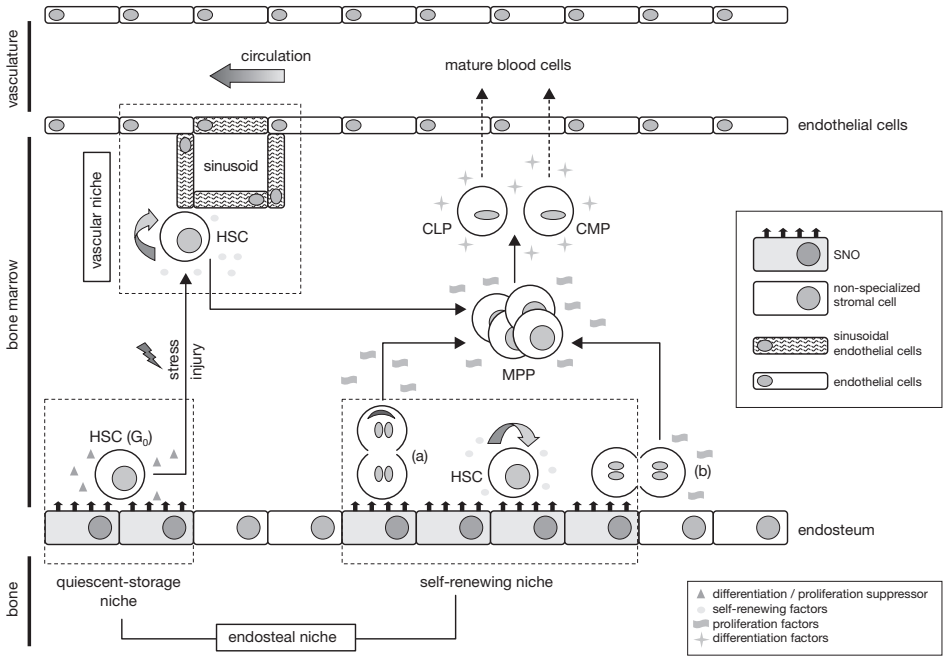

Figure 4. Current model of the bone marrow HSC niches

The cellular barrier between the bone and bone marrow is formed by the endosteum, which consists of non-specialized stromal cells and SNOs (Spindle-shaped N-cadherin-expressing Osteoblasts). The SNOs create the two different types of niches. Within these niches the HSCs are though to be anchored via homotypic N-cadherin interactions. The quiescent-storage niche anchors HSCs within G_0-phase. Upon stress and/or injury, this pool of quiescent HSCs can be activated to help boost the re-establishment of the hematopoietic system. The self-renewing niche mainly contains activated HSCs, which are able to self-renew and differentiate. Asymmetric differentiation can occur via pre-cell division (a) or post-cell division (b) generating multipotent progenitors (MPPs). Within the bone marrow are vascular sinusoids which have been shown to contain another HSC niche; the vascular niche. The HSCs in this niche will also differentiate towards MPPs as they can directly react to changes in the hematopoietic system. Finally, the MPPs will differentiate along the adult hematopoietic hierarchy to mature blood cells, which are released in the circulation. The niches, via ligand-receptor interactions, adhesion molecules and cytokine expression, control all the different steps in the maintenance, differentiation and proliferation of HSCs [122,196].

tion is in the self-renewal and differentiation of HSCs via asymmetric division. The self-renewing niche is thought to be the essential unit for maintaining the equilibrium of the hematopoietic system. After HSC division one daughter cell will differentiate producing progenitors and eventually mature hematopoietic cells, while the other daughter retains HSC properties. The differentiating daughter cell tends to lose its attachment to the niche-cells, while the HSC daughter stays firmly attached [122,196]. The self-renewing and quiescent niches are thought to be located at different places within the adult bone marrow.

1.3.1 The endosteal bone marrow HSC niche

It was first shown in the 1970s that hematopoiesis and osteogenesis (bone development) are closely linked. Patt and co-workers [144] showed that after the bone is formed, resorption is required to form the vascularized bone marrow harbouring the hematopoietic cells. Recent studies revealed that mice defective in hematopoiesis may have a primary defect in bone development and/or remodelling. For example the CBFα1 (Runx2) knockout mice [31,78,142] have a decreased number of HSCs. In this mouse model no osteoblasts, no bone or bone marrow are being developed, thus resulting in the absence of a hematopoietic system. These studies provided the first *in vivo* evidence of the involvement of osteoblasts in the niche formation. The opposite also holds true. For example, an increased number of HSCs was shown in the conditional bone morphogenetic protein receptor type IA (BMPR1A) knock-out mouse [211]. This is thought to be a consequence of the increased number of osteoblasts in these mice. These models strongly suggest that the osteoblasts within the bone marrow are essential in the formation of the HSC niche.

Osteoblasts are part of the endosteum in the bone marrow. This cell layer separates the bone from the bone marrow and also contains stromal fibroblasts and endothelial cells (Figure 4). Using the previously mentioned BMPR1A knockout mouse model, Zhang and co-workers showed by 5-bromo2'-deoxy-uridine (BrdU) labelling that LTR-HSCs localize to the endosteum lining. In this study they made use of the quiescent nature of LTR-HSCs. This enables these cells to retain labelled nucleotides like BrdU. This specific location harbouring LTR-HSCs was named the endosteal HSC niche of the bone marrow. Committed hematopoietic progenitors were found in the central bone marrow region, more distant from the endosteal lining [55,165,195,211].

In vitro evidence for the role of osteoblasts in the HSC supporting activity came from studies involving stromal cell lines. First, osteoblast cell lines produce cytokines, which promote proliferation of hematopoietic cells in culture [179]. Some of these cytokines are IL-1, GM-CSF, TNF-α and IL-6. Second, long-term bone marrow cultures containing osteoblasts and stromal cell lines are able to maintain HSCs *in vitro* [132,179]. Furthermore, the mouse model generated by Calvi and co-workers not only demonstrated an increased number of osteoblasts but also showed an increase in HSCs [17]. Stromal cells isolated from the bone marrow of these mice showed an overall better support of HSCs in *in vitro* co-cultures compared to wild type stromal cells. This was thought to be due to the elevated number of osteoblasts in the stromal cell population.

Additionally, Corral and co-workers [23] determined that niches in the bone marrow are comprised of immature osteoblasts. They used a mouse model where the expression of thymidine-kinase was under the control of an osteocalcin promoter, which restricts expression to mature osteoblasts. Upon gancyclovir treatment all mature osteoblasts died. Hematopoiesis in these mice was unaffected, suggesting a role for the osteoblast progenitors in the formation of the HSC niche.

Overall, the endosteal HSC niche is thought comprise of more than osteoblast progenitors. Osteoclasts, stromal fibroblasts and endothelial cells within this structure can also contribute to the niche formation and activity. At the endosteal lining it is thought both the quiescent and the self-renewing niche types are present, forming different endosteal HSC niches (Figure 4).

1.3.2 The vascular bone marrow HSC niche

Since it has previously been shown that exogenous cytokines, when administered *in vivo*, are able to mobilize HSCs into the circulation within minutes [83], it was suggested that HSCs reside very close to the blood vessels within the bone marrow [59]. It was suggested that this type of niche harboured HSCs, until they were activated; i.e. upon stress induction. This newly suggested niche is thought be located at the endothelium of the bone marrow sinusoids (Figure 4). Since there is a suggested close relationship between HSCs and endothelial cells at early developmental stages [70], a niche comprised of endothelial cells seems logical. This is supported by the *in vitro* maintenance of HSCs using endothelial-derived cell lines. However, this is only a subset of endothelial cells since endothelial cells from non-hematopoietic tissues showed little support for HSCs *in vitro* [90].

Recently Kiel and co-workers [77] set out to identify more specific markers characteristic of HSCs. Using gene expression profiling techniques they found that LTR-HSCs express the homotypic cell surface receptor CD150 at significantly higher levels compared to more differentiated hematopoietic cells. Using this newly identified marker in immunostaining experiments of bone marrow samples, they found $CD150^{+}$ LTR-HSCs to be attached to the sinusoidal endothelium. Thus the now-called vascular HSC niche was identified. The presence of such a vascular HSC niche in the adult bone marrow at the same time as the endosteal HSC niche suggests that both are present during maintenance of the hematopoietic system [77]. Existence of the vascular HSC niche allows HSCs to monitor the signals in the circulation indicating the current state of the hematopoietic system. The HSCs in the vascular HSC niche are therefore thought to be involved in homeostasis and to be self-renewing activated rather than long-term quiescent HSCs. Evidence that the vascular HSC niche exhibits the molecular properties of self-renewing HSC niche located at the endosteum is still lacking.

1.3.3 Regulation of HSCs in the niche

As mentioned previously, two types of niches coexist within the bone marrow, the endosteal and vascular niches [91,206]. It is thought that the bone marrow niches are able to maintain quiescence and self-renewal capacity of HSCs and to activate HSCs to enter proliferation and differentiation programs. This occurs through a tightly regulated cross-talk between niche cells and HSCs. Pathways involved in regulation of developmental processes are thought to contribute to this cross-talk and include the Wnt-, BMP-, Notch-, Hedgehog- and FGF-signalling pathways [91,206].

At any given point in time most (~80%) HSCs in the bone marrow are quiescent [47]. Upon activation by stress or injury, the HSCs quickly respond to restore the equilibrium of the hematopoietic system. Quiescent HSCs are thought to reside mainly within the endosteal niche [91]. Maintaining quiescence of HSCs within the niche is largely dependent on cell-cell contact between niche-cells and HSCs. Previously it was reported that the activation of the Notch-signalling pathway maintains HSCs and keeps hematopoietic progenitors in an undifferentiated state [88,191]. Calvi and co-workers found that Notch is expressed by HSCs and its ligand, Jag1, is expressed by osteoblasts and bone marrow stromal cells [17]. Via this receptor-ligand binding, HSCs remain quiescent and are tightly anchored to the niche. Another receptor-ligand interaction was implicated in anchoring HSCs to the niche when Aria and

co-workers showed that quiescent HSCs firmly attached to osteoblasts in the endosteal niche via the receptor tyrosine kinase Tie-2 [4]. The Tie-2 receptor present on HSCs is activated by the production of angiopoietin-1 (Ang-1) by the osteoblasts. This activation promotes tight adhesion of the HSCs to the niche, resulting in the maintenance of HSC quiescence and the prevention of HSC division. More recent *in vivo* data implicated the Ca^{2+}-sensing receptor (CaR) in facilitating retention of HSCs within the endosteal niche [1]. Mice deficient in CaR release HSCs into the bloodstream instead of anchoring them in the niches. Furthermore, Zhang and co-workers showed the importance of adherens junction molecules, namely N-cadherin and β-catenin [211]. These adhesion molecules provide anchors for HSCs to stay attached to the niche-cells, inhibiting proliferation and differentiation. Moreover, by increasing the number of osteoblasts it was shown that Notch-signalling was enhanced and the HSCs pool expanded [17]. By increasing the number of osteoblasts expressing N-cadherin (SNOs; see Figure 4), or by disturbing BMPR1A, HSCs also increase [211]. These studies suggest that the size and/or number of niches correlate with the HSC numbers. Furthermore, a link between adhesion molecules and cell-cycle regulators in maintaining HSC quiescence was made by identification of N-cadherin as a target of Tie-2/Ang-1 signalling pathway [4].

Upon activation of HSCs to restore the equilibrium of the hematopoietic system, proliferation and differentiation must occur without the loss of all HSCs. Asymmetric cell division of HSCs plays a role in preserving adequate numbers of these cells. Adhesion molecules, like N-cadherin, β-catenin and VCAM, are thought to play a role in the asymmetric production of progeny by facilitating anchoring of HSCs to osteoblasts after and during division. A key player in regulating the levels of N-cadherin and other intergrins within the HSCs is c-Myc [122,195]. Upon HSC division a daughter cells can express low levels of c-Myc, leading to an upregulation of N-cadherin. This daughter cell will be anchored in the niche via the N-cadherin interactions preserving HSC properties. In contrast, when high levels of c-Myc are present, there is a down regulation of N-cadherin allowing the daughter cell to leave the niche for further proliferation and/or differentiation [195].

It was suggested that the vascular niche facilitates HSC migration during homing or mobilization. Furthermore, this niche seems to favour HSC proliferation and differentiation. Recruitment of HSCs to both types of niches depends largely on chemokines like stromal-derived-factor 1 (SDF-1) and fibroblast growth factor 4 (FGF4). Endothelial cells, osteoblasts and other stromal cells express SDF-1 and HSCs express its receptor CXCR4 [79]. SDF-1 is mostly present at the endosteal niche and thus CXCR4 expressing HSCs are most likely attracted to this niche via the receptor interacting with SDF-1 [91,206]. Lowering the levels of SDF-1 at the endosteal niche leads to an increase of HSCs in the circulation. G-CSF, presently used to mobilize HSCs for transplantations, primarily decreases SDF-1 levels in the osteoblasts and increases SDF-1 in the circulation. The decrease of SDF-1 in the osteoblasts occurs through G-CSF induced expression of proteolytic enzymes, like elastase and matrix metalloproteinase 9 (MMP9). Proteolytic cleavage of SDF-1 by these enzymes leads to its inactivation. Upon stress SDF-1 plays an additional role together with VEGF. A disturbed balance of the hematopoietic system could lead to an induction of MMP9 via SDF-1 and/or VEGF. MMP9 will convert the membrane-associated Kit ligand into a soluble Kit ligand (sKitL). This release of the receptor promotes HSCs to enter cell

cycle and start proliferation and/or differentiation. Thus, SCF/c-kit receptor-ligand interactions are implicated in the control of HSC activation and release from the niche [59].

In the past a number of molecules and signalling pathways were implicated in HSC regulation. Wnt-signals are important in regulating self-renewal [152,173], yet expression of molecules of this signalling-cascade within the niche is unknown. The same holds true for FGF and Hedgehog signalling. Both pathways affect HSCs *in vitro* [10,30]. HSCs are known to express FGF-receptors and upon stimulation they will proliferate and self-renew *in vitro*. Another indication of FGFs being involved in the niche cross-talk with HSCs is the observation of a FGF4 gradient [6]. High levels of FGF4 are present at the vascular niche influencing recruitment, proliferation and differentiation of HSCs [79,91,206].

Currently, many studies are ongoing attempting to reveal more details on how HSCs communicate with their surrounding niche-cells, whether more types of niches exist (in different tissues), how many HSCs are present within the niches and how dynamic the interactions really are.

1.3.4 HSC niches in the embryo

It was suggested that during development the emerging HSCs need a microenvironment for their maintenance similar to that in the bone marrow. Determining the presence of such niches within the embryo was mainly performed using cell lines, an *in vitro* equivalent of the *in vivo* niches. As described before, this technique was used previously to analyse the bone marrow niches. Already in the 1990s one of the first attempts to determine the presence of such environments within the embryo was undertaken by Moore and co-workers [117]. They generated a fetal liver derived stromal cell line (AFT024), which supports LTR-HSCs efficiently. In addition, Yoder and co-workers [207] showed that endoderm and mesoderm derived cell lines from the yolk sac also support hematopoietic cells efficiently. The most potent region during embryonic development, where the first HSCs are emerging, is the AGM region suggesting that the AGM harbours a very potent microenvironment for HSCs. Stromal cell lines from the AGM region were generated and tested for the ability to support HSCs and progenitors. Two of the first AGM derived cell lines, the AGM-S3 [199] and DAS104-4 [129] were able to maintain hematopoietic progenitors and HSCs *in vitro*, respectively. To study more closely the supportive capacities of the AGM region Oostendorp and co-workers [132] generated over 100 cloned stromal cell lines from sub regions of the AGM. Hereby the AGM was divided into the aorta-mesenchyme (AM) and the urogenital-ridges (UG) regions. Several of the generated cell lines showed efficient *in vitro* support of HSCs from the bone marrow and AGM region. The best supporting cell line was derived from the urogenital-ridges (UG26-1B6) but cell lines derived from the aorta-mesenchyme region also supported HSCs and progenitors. In comparison to cell lines from the fetal liver and bone marrow [133] it was shown that the AGM derived cell lines are more potent in supporting HSCs and progenitors.

Cells comprising the adult HSC niches are of mesenchymal origin, e.g. the osteoblasts [32]. Recent data showed that mesenchymal progenitors are present at the major hematopoietic sites during mouse embryonic development [105]. The presence of the mesenchymal progenitors seems to be independent of the presence of HSCs. This was indicated using *Runx1* -/- AGM regions (lacking normal hematopoiesis; see chapter 1.3.6 for detailed explanation of Runx1 and HSCs) where the number of different mesen-

chymal progenitors was found to be similar to wild type AGM regions. Further studies on the function of the microenvironment in the AGM region were performed by Harvey et al. [58] who indicated that cell-cell contact is essential for maintenance of embryonic derived LTR-HSCs *in vitro*. The presence of soluble signalling molecules, analysed by non-contact co-cultures, supported only hematopoietic progenitors. This study also indicated that HSCs derived from similar developmental and anatomical locations, as the cell lines are the most potent combinations in maintaining HSCs and progenitors *in vitro*. So, AGM derived HSCs are best maintained on AGM derived cell lines.

1.3.5 Growth factors and Cytokines

Growth factors and cytokines play important roles in the hematopoietic system during ontogeny. In the 1960s a cell culture system (CFU-C), allowing blood lineages to differentiate, provided a tool to identify, purify and clone cytokines and growth factors involved in the regulation of hematopoiesis [53]. Using this approach several colony-stimulating factors (CSFs) were identified. These factors when present in the medium of cell culture systems were able to induce the growth (leading to clones/colonies) and maintain the viability of the hematopoietic cells in culture, as well as inducing differentiation to several hematopoietic lineages. Several of the first identified CSFs are; macrophage-CSF (M-CSF), granulocyte-CSF (G-CSF) and granulocytic/macrophage-CSF (GM-CSF). Furthermore, IL-3 was discovered to affect macrophages, granulocytes, eosinophils, mast cells, erythroid cells and megakaryocytes and thus was initially thought to be the multipotency or stem cell factor [94]. These primary studies lead to the isolation of more and more cytokines and growth factors involved in hematopoiesis, e.g. SCF (or kit-ligand) [198], Flt3/Flk2 ligand (FL) [97] and thrombopoietin (TPO) [75]. Moreover, hematopoietic cells were found to express different receptors on their surface permitting specific interactions with the cytokines and growth factors [57]. Signalling pathways are activated via binding of these factors to the receptors leading to expression and/or repression of an array of downstream target genes.

1.3.5.1 Fibroblast Growth Factors and hematopoiesis

Fibroblast Growth Factors (FGFs) are a large family of molecules found in all vertebrate species that are important for cell development and growth. Currently, there are 22 known structural related members grouped in subfamilies [137,178] (Table 2). All FGF members share a conserved central domain of about 120 amino acids, which facilitates the interactions with their receptors. This domain also binds to heparin [151,184,204], which will stabilize the FGF-FGF receptor (FGFR) interactions.

Most FGFs contain a terminal signal sequence that directs immediate secretion from the cells. However, some FGFs lack this terminal signal sequence and can be either secreted (FGF9, 16, 20) or not (FGF1, 2) [112,113,128]. FGF1 and 2 are both found on the cell surface as well as within the extra cellular matrix and are suggested to be released via a pathway independent of the Endoplasmic Reticulum-Golgi complex [107]. The FGF11 subfamily, is not secreted but are thought to remain intracellular [121,167]. FGFs are differentially expressed in many tissues temporally as well as spatially. However, within subfamilies there tends to be overlap in time and pattern of expression.

FGFRs are members of the tyrosine kinase receptor family expressed from four different related

Table 2. FGF subfamilies, FGFs and their features
(Adapted from: [43,137,138,187,213] and OMIM-PubMed database)

Subfamily	FGF	FGFR activity @	Mouse mutant phenotypes	FGF function
FGF1 subfamily	FGF1	all FGFRs	viable, no obvious phenotype	modifier of endothelial cell migration and proliferation universal FGFR ligand
	FGF2	FGFR 1c, 3c (2c, 1b, 4)	viable, neuronal, skeletal and skin phenotypes	wide-spectrum mitogenic, angiogenic, and neurotrophic factor expressed at low levels in many tissues and cell types high concentrations in brain and pituitary
FGF4 subfamily	FGF4	FGFR 1c, 2c (3c, 4)	lethal (E5.5) inner cell mass proliferation	potential oncogene, important in tooth-development linked to the Wnt-signalling
	FGF5	FGFR 1c, 2c (3c, 4)	viable, long hair phenotype	expressed in neonatal brain, inhibitor of hair elongation
	FGF6	FGFR 1c, 2c (3c, 4)	viable, muscle regeneration	potential oncogene
FGF7 subfamily	FGF3	FGFR 2b (1b)	viable, inner ear tail outgrowth	role in development of the inner ear and lung cancer
	FGF7	FGFR 2b (1b)	viable, hair follicle and kidney deficiency	affects epithelial cells and keratinocytes expressed by fibroblasts
	FGF10	FGFR 2b (1b)	lethal (P0), many phenotypes e.g. limbs, lungs, kidneys etc.	expressed developing epidermis and hair follicles
	FGF22	FGFR 2b (1b)	na	preferential expression in skin
FGF8 subfamily	FGF8	FGFR 3c (4, 2c, 1c, 3b)	lethal (E8.5), many phenotypes gastrulation, brain, heart, craniofacial development	expressed in testis but not in other adult tissues, expressed in embryos at E9 through E13 only
	FGF17	FGFR 3c (4, 2c, 1c, 3b)	viable, midline, cerebral development	expression is dependent on sonic-hedge hoge preferentially expressed in the embryonic brain
	FGF18	FGFR 3c (4, 2c, 1c, 3b)	lethal (P1), delayed ossification and increased chondrocyte proliferation decreased alveolar spaces in lung	abundant expression in lung and kidney (adult) induces proliferation primarily in liver and small intestine and osteoblasts and chondrocytes
FGF9 subfamily	FGF9	FGFR 3c (2c, 1c, 3b, 4)	lethal (P0), lung, XY sex reversal	stimulate mesenchymal proliferation and mesonephric cell migration
	FGF16	FGFR 3c (2c, 1c, 3b, 4)	disturbed cerebellar development	expression in heart
	FGF20	FGFR 3c (2c, 1c, 3b, 4)	'devoid of blastema' (dob) mutant (zebrafish), fails fin regeneration and does not form blastema	transcript expression in colon cancer cell line, SW480 low levels expression in fetal brain, fetal liver, fetal kidney
FGF19 subfamily	FGF19	FGFR (1c, 2c, 3c, 4)	na	expressed only in fetal brain mediator of mesodermal signals
	FGF21	FGFR (1c, 2c, 3c, 4)	na	high expression in liver and weaker expression in thymus metabolic factor with therapeutic characteristics
	FGF23	FGFR (1c, 2c, 3c, 4)	viable, hyperphosphatemia hypoglycemia, reduced bone density and infertility	high expression in brain and lower expression in thymus
FGF11 subfamily (FGF homologous factors; FHFs)	FGF11	no known activity	na	FGF Homologous Factor 3; FHF3
	FGF12	no known activity	na	FGF Homologous Factor 1; FHF1
	FGF13	no known activity	na	FGF Homologous Factor 2; FHF2 highest expression in brain and skeletal muscle
	FGF14	no known activity	viable, neurological phenotype-ataxia and paroxysmal hyperkinetic movement disorder	FGF Homologous Factor 4; FHF4

@ = activation of receptors through ligand; between brackets indicates weak receptor activity

genes; FGFR1-4. The FGFRs are comprised of an intracellular tyrosine kinase domain, a single transmembrane domain and an extracellular ligand binding domain [212]. The extracellular domain contains two or three immunoglobulin (Ig)-like domains, which can be alternatively spliced. Splicing of the Ig-like domain III can result in three different variants (named: IIIa, IIIb and IIIc) that can differentially bind to FGF species [138]. The splice variant IIIa forms a secreted extracellular FGF-binding protein. The signalling capacity of the IIIa isoform is not yet known [38]. The IIIb and IIIc splice forms are regulated in a tissue specific manner; IIIb is restricted to the epithelial lineages whereas the IIIc is preferentially expressed in mesenchymal lineages [212].

Binding of two FGFs-heparin complexes to their FGFRs results in receptor homodimerization that triggers tyrosine kinase activation. This then leads to autophosphorylation of the intracellular domains. From there onwards FGF signal transduction can proceed via three main pathways, the Ras/MAPK pathway, the PLCγ/Ca^{2+} pathway and the PI3 kinase/Akt pathway. These pathways have previously been implicated in various kinds of cellular responses like gene transcription, induction of cell death, maintenance of cell survival, cell growth and differentiation [15]. The most common pathway employed by FGFs is the Ras/MAPK pathway (reviewed by [12]), which leads to a phosphorylation of transcription factors, such as c-myc, AP1 and members of the Ets family [85,185].

As indicated at the beginning of chapter 1.3, the bone marrow microenvironment seems to be important for the maintenance of the hematopoietic system during adult life, linking bone development to hematopoiesis. In adult bone marrow, stromal cells of the microenvironment play part in regulating the balance between HSC and progenitor self-renewal and proliferation/differentiation. In the 1990s mutations in the transmembrane domain of FGFR3 were discovered resulting in achondroplasia, the most common genetic form of dwarfism in human [156,163]. This led to intensive research suggesting that FGFs and FGFRs play a major role in many human bone-related disorders, which ultimately could also affect the hematopoietic niches. FGFs implicated in bone formation are FGF2 and FGF18, which are expressed in mesenchymal cells and osteoblasts. Over expression of FGF2 in mouse induced abnormal bone formation [21], whereas FGF2-knockout mouse showed inhibition of bone formation [116]. FGF18 induces proliferation in osteoblasts and chondrocytes (Table 2). As FGFs, FGFRs are also differentially expressed during bone formation regulating the actions of FGFs. In early bone development FGFR1 and FGFR2 are expressed on mesenchymal cells whereas later in development they are co-expressed together with FGFR3 in osteoblasts and their progenitors [115]. Thus, FGF signalling appears to play a role in bone development, more specifically at the stages during osteoblast formation.

Within the bone marrow stromal microenvironment, the extracellular matrix is thought to serve as a reservoir of growth factors and cytokines [46]. FGF2 has been found to remain in the bone marrow extracellular matrix [46] and to be a potent mitogen for bone marrow stromal cells *in vitro* [3,131]. In addition, FGF2 may also directly affect hematopoietic cells [8,197,210]. Using human long-term bone marrow cultures FGF4 appeared to affect the stromal cells resulting in higher numbers of CFU-Cs present in these cultures, although an effect of FGF4 directly on hematopoietic progenitors could not be ruled out [150]. Extensive work on *Xenopus* embryos showed that ventral injections of FGF suppress ventral blood island formation [200]. Previous studies by Bartunek and co-workers [8] identified an erythroid progenitor

that is critically dependent on FGF2. They also suggested that FGF2, in cooperation with Myb proteins, represent an important factor for determining erythroid lineage choice.

FGFRs are expressed on almost every cell type of hematopoietic origin [3,30]. To date, LTR-HSCs are found to express FGFRs, which will be lost upon differentiation towards more committed hematopoietic progenitors [30]. Using FGF1 as the only stimulatory growth factor in long-term bone marrow cultures, de Haan et al. [23] observed an expansion of hematopoietic progenitors and LTR-HSCs. These results indicated that properties of HSCs are dependent on signalling through FGFRs and that FGF-1 plays an important role [30]. Since LTR-HSC activity is maintained in the FGFR expressing cell population of the bone marrow [30], Yeoh and co-workers suggested that stimulation of the FGF signalling pathways through other FGF family members may be important in hematopoiesis [205]. Interestingly, they showed that FGF2 also plays a role in supporting of LTR-HSC *in vitro*. Moreover, they suggested that effects of FGFs on HSCs also rely on other stimuli, since *in vitro* cultures of enriched HSCs populations with FGF1 and/or 2 where unable to maintain the multipotential properties of HSCs.

Since HSCs and progenitors appear during embryonic development, it has been suggested that there are temporal microenvironments allowing emergence, expansion or maintenance of HSCs. Evidence for this mainly comes from the study of stromal cell lines generated from the different hematopoietic tissues. Stromal cell lines from fetal liver, yolk sac and AGM support hematopoietic cells *in vitro* [117,132,207]. Blood vessels play an important role in the development and maintenance of the hematopoietic system. The first LTR-HSCs are localized at the major vessels within the embryo. Recent studies indicated that FGF signalling plays a role in angiogenesis by effecting endothelial cells (reviewed in [149]). The first indication of FGF signalling involved in the AGM microenvironment comes from the detection of elevated levels of *Fgf7* and *Fgf9* transcripts in AGM cell lines providing HSCs support *in vitro* [134]. Currently, more studies are attempting to elucidate the role of FGF signalling in the hematopoiesis at embryonic stages as well as adult.

1.3.5.2 Interleukin-1 and hematopoiesis

A large number of growth factors are known to be effectors of hematopoiesis. This includes interleukins (25 interleukins identified so far), CSFs and tumour necrosis factor (TNF). Interleukins were first detected in white blood cells (leukocytes), which use them to communicate. Hence the name; inter- (communication) leukin (leukocytes). The function of the immune system depends largely on interleukins. Interleukin-1 (IL-1) is a member of this large family of cytokines. It is expressed in many types of cells such as monocytes, neutrophils, endothelial cells and fibroblasts. IL-1 is an important part of the inflammatory response of the body against infections. By stimulating an increase in the adhesion factors on endothelial cells, IL-1 induces neutrophils to migrate to the infection sites [119]. Two IL-1 isoforms have been identified; IL-1α and IL-1β. The activity of these isoforms is regulated both at the transcriptional and posttranslation levels [35]. Both isoforms are produced as precursor proteins, needing proteolytic cleavage to become active [358].

The IL-1R receptor is composed of two subunits, the IL-1RI and the IL-1RAcP (associating protein). The IL-1 protein binds to the IL-1RI, after which the IL-1RAcP is recruited. IL-1RAcP stabilizes the

complex and mediates recruitment of downstream signalling molecules [39]. Another IL-1R type, IL-1RII was found to lack an intracellular signal-propagating domain. This very short receptor is unable to recruit signalling molecules upon binding of IL-1. An inhibition of the activity of IL-1 via this decoy IL-1RII is another mechanism of regulation [22,39]. In addition, while IL-1RI and IL-1RII are integrated in the cell membrane, proteolytic cleavage of these receptors results in two soluble receptor forms. These soluble IL-1Rs can still bind IL-1, but will inhibit the downstream signalling pathway activation, since they are no longer able to recruit signalling molecules at the cell membrane. As a result IL-1 activity is silenced [39,168].

The IL-1Rs belong to the IL-1/Toll like receptor superfamily. A common feature of this superfamily of receptors is the presence of an intracellular Toll-IL-1 receptor domain (TIR-domain) [39]. This domain is suggested to play a crucial role in the signalling transduction [166] through MAP-kinase pathway, JNK pathway and the NFκB signalling cascade [35,36,39,164]. JNK and NFκB are regulated via recruitment of the IRAK kinases to the IL-1/IL-RI/IL-1RAcP complex. Second, the kinase TAK1 propagates the downstream signal to JNK and NFκB pathways via the adaptor proteins TRAF6, TAB1 and TAB2.

Osteoclast activity is stimulated by both isoforms of IL-1. Osteoclasts are increased in number, thus leading to an increased bone resorption [157]. They are released as a consequence of cell injury leading to apoptosis [61]. In the past, numerous studies showed that IL-1 signalling could affect the expression of cytokines (and their receptors), inflammatory mediators, growth factors, clotting factors, oncogenes, adhesion molecules and extracellular matrix molecules [35]. By injecting mice with recombinant IL-1, Fibbe and co-workers [45] showed that IL-1 releases HSCs and progenitors in a dose-dependent manner into the peripheral blood. Upon IL-1 stimulation, a shift in cell adhesion of these cells in the bone marrow can be achieved. IL-1 is therefore implicated in cell adhesion and migration processes. Functional studies showed that IL-1 acts as a radioprotector of HSCs and progenitors [123,135,214] and was shown to be involved in expansion of hematopoietic progenitors and myeloid precursors [14,44]. Furthermore, IL-1 and IL-1Rs are expressed in HSCs and progenitors [66,100,102]. In contrast, other studies showed that IL-1 induced differentiation of hematopoietic progenitors resulting in loss of HSC activity [68,208].

The role of IL-1 during the embryonic development of the hematopoietic system is still under investigation. Recently, Orelio and co-workers [136] determined TAB2 protein to be up regulated between E10 and E11 in the AGM region of a mouse embryo. The expression pattern of TAB2 in the dorsal aorta endothelium correlates with the emergence of HSC activity in this region. TAB2 was identified as a binding partner for TAK1, which plays a crucial role in IL-1 signalling pathway. Involvement of the IL-1 signalling pathway in the emergence and/or regulation of HSCs during development were suggested.

1.3.6 Transcription factors

Transcription factors play an important role in the way signals are translated into cellular behaviour. Transcription factors are classified by DNA binding motifs into families. Within these families transcription factors are expressed in a cell specific and/or developmental stage specific manner, thus regulating cell fate, differentiation and proliferation. Also during embryonic development and adult

life, the levels of transcription factors are important for maintaining the self-renewal of stem cells. A slight change in the levels and/or spatial and temporal expression pattern of transcription factors, could lead to a disturbance of this balance subsequently leading to defects in an entire tissue or system, such as the hematopoietic system in leukaemic syndromes.

In normal development of the hematopoietic system, several transcription factors have been shown to be pivotal to the emergence and/or regulation of HSCs. These include the Runx1 [130,193], GATA2 [186] and SCL/Tal1 [148,154] transcription factors. Briefly, GATA2 is expressed in immature hematopoietic cells and upon differentiation GATA2 expression is down-regulated [145]. Mice deficient for GATA2 die between E10.5 and E11.5 due to anaemia [186]. Analysis of these mice showed that they have a reduced number of primitive erythrocytes. Furthermore, definitive hematopoiesis was disrupted. It was concluded that GATA2 expression is required for all hematopoietic lineages. The SCL/Tal1 transcription factor is expressed in the developing blood islands of the yolk sac at E8.5, within the endothelial and the hematopoietic cells. Expression of SCL/Tal1 is also found in the fetal liver between E10.5 and E14.5. The increased expression of SCL/Tal1 in the fetal liver correlates with increased hematopoietic activity [74]. Mice deficient in SCL/Tal1 died around E9.5 due to absence of yolk sac hematopoiesis and abnormalities in the vitelline vessel [155]. Suggestions were made that SCL/Tal1 deficiency leads to an early block in hematopoietic development. Because studies presented in this thesis have focused on a role for Runx1 in the hematopoietic microenvironment, the Runx1 transcription factor and its role in hematopoiesis will be described in more detail in the next section.

1.3.6.1 The Runx family of proteins

Runx family members are the alpha-subunits of the heterodimeric Core Binding Factor (CBF) complex. Dimerization with the beta-subunit (CBFβ) stabilizes the complex and enhances the DNA-binding properties. The Runx proteins bind to the DNA through their Runt homology domain (RHD), which is evolutionarily conserved from *Drosophila* to humans. Presently, three mammalian homologues are identified, Runx1, Runx2 and Runx3 (Table 3 and Figure 5). All three Runx genes encode several isoforms due to alternative mRNA splicing and different promoter usage (each gene is transcribed from two promoters) [86]. Expression pattern analysis of the three Runx proteins and the CBFβ protein show their presence in bone marrow, thymus and peripheral lymphoid organs. They display some overlapping but different expression patterns [27].

So far, little is known about the factors and/or pathways controlling the expression of the three Runx proteins. Drissi and co-workers showed that within the promoter regions of the three Runx proteins, binding sites for Runx proteins are present [37], suggesting that autoregulation of the Runx proteins occurs. Whether the individual Runx proteins do indeed regulate their own expression and/or the expression of family members is still under investigation. The only established regulation of the Runx proteins is by retinoids [72,84,181]. An increased level of retinoic acid leads to an increased expression of Runx1 and Runx3, but decreases the Runx2 levels. Runx proteins are essential in different processes. Runx1 is implicated in hematopoiesis; Runx2 in bone ossification and Runx3 in the gastrointestinal tract and nervous system [78,87,89,142]. Runx proteins exert distinctive developmental roles (Table 3), which

Table 3. Runx protein family members and their features
(Adapted from: [11,27,95,190])

Name:	Runx 1	Runx 2	Runx 3
Synomyms:	CBFA2, AML1, PEBP2alphaB	CBFA1, AML3, PEBP2alphaA	CBFA3, AML2, PEBP2alphaC
Locus location: Human Mouse	 21q22 16 (62.2 cM)	 6p21 17 (28.07 cM)	 1p36 4 (65.7 cM)
Essential function:	- Definitive hematopoiesis	- Bone ossification	- Development of gastrointestinal tract and the nervous system
KO phenotype in mouse:	- Embryonic lethal (E12.5) - Absence of FL hematopoiesis - Absence of intra-aortic hematopoietic clusters - Early appearance of HSC in AGM region - Reduced number of LT-HSCs	- Lethal at birth owing to respiratory failure - Failure of osteoblast differentiation and bone formation - Reduced numbers and function of osteoclast-like cells	- Lethal within 10 days after birth (75% died the first day) - Hyperplastic gastric ephithlium due to excessive proliferation - Loss of dorsal-root ganglion proprioceptive neuron function - Loss of homeostatic control of dendritic cell function - Lung inflammation
Diseases in human:	- ~30% of leukemias - Familial platelet disorder - Predisposition to AML	- Autosomal dominant bone disease (CCD = cleidocranial dysplasia)	- Gastric cancer

most likely arise from tissue-specific control of their expression. This is supported by the observation that the hematopoietic defects resulting from Runx1 deficiency can be rescued by knock-in of parts of the coding exons of Runx2 and Runx3 into the Runx1 locus [56]. To further understand why the three highly related family members display such different functions, analysis of the upstream regulators is necessary.

In vitro analyses by transcriptional reporter assays indicate that all three family members have identical effects on target promoters [71]. The runt domain is able to specifically bind to a consensus sequence 5'-PuACCPuCA-3'. As indicated before, Runx proteins are able to activate and/or repress

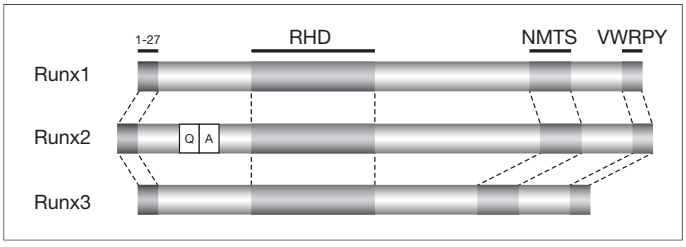

Figure 5. The structure of mammalian Runx proteins
The family of Runx proteins consists of three members, Runx1, Runx2 and Runx3. The Runt-homology domain (RHD) is the most conserved feature of these proteins. Other conserved domains are the nuclear-matrix-targeting signal (NMTS) and the carboxy-terminal VWRPY motif. The first 27 amino acids are also conserved between family members. An extra feature only present in Runx2 is an extended stretch of glutamine (Q) and alanine (A). At the C-terminal site of the RHD a proline, serine and threonine-rich region (PST-region) is located. Runx1 and Runx2 both harbour a transactivation domain just before the NMTS [7,11,65].

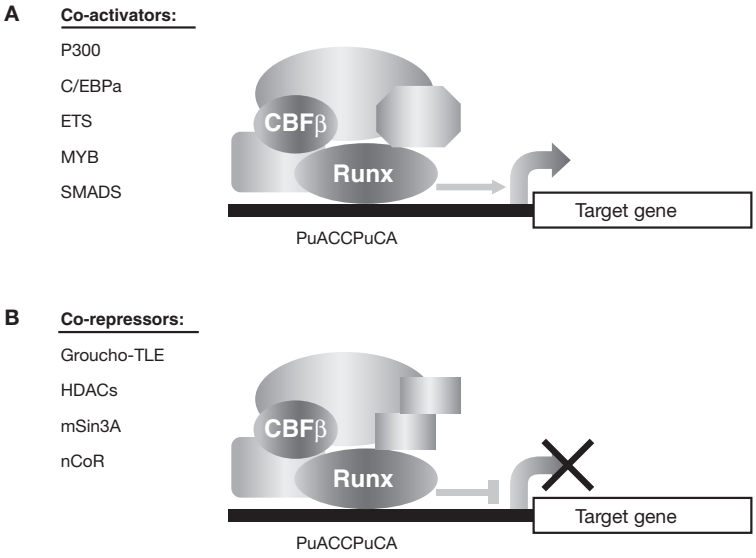

Figure 6. Runx proteins and target gene expression/repression
Runx proteins bind to their target sequence (5'-PuACCPuCA-3') in concert with CBFβ to form the CBF-complex. Target gene expression **(A)** or repression **(B)** can be achieved by recruitment of non DNA-binding proteins (p300, mSin3A, HDAC, TLE and nCoR) to the CBF-complex. Cooperation between lineage-specific transcription factors (C/EBPa, ETS and Myb) that bind adjacent to the Runx consensus often leads to expression of the target genes. Whether Runx proteins act as activators or repressors of target gene expression can be dependent on several factors, including the acetylation and/or phosphorylation of the Runx proteins. Also the availability of co-factors in the nucleus may determine whether Runx proteins express or repress target genes [11,81,96].

transcription (Figure 6). To activate gene expression Runx proteins within the CBF complex will interact with coactivators like histone acetyltransferases (p300/CBP), C/EBPα, ETS family members and SMADS. Target gene repression is established by recruiting corepressors e.g. Groucho homologue TLE, HDACs and mSin3a [40]. Downstream target genes can therefore be up- or down regulated, depending on the presence of the activator and/or repressor in the nucleus (Figure 6) [141]. Several studies suggest that Runx proteins are post-translationally modified by phosphorylation [182,194] and acetylation [73,202]. These modifications are thought to actively modulate the switch between repression and activation of target genes [49,64]. While many studies have been performed since the discovery of Runx proteins, most Runx target genes have been studied in the hematopoietic (Runx1) and bone/cartilage (Runx2) compartments.

1.3.6.2 Runx and hematopoiesis

In the 1990s it was discovered that *AML1*, the human homologue of *Runx1*, is a gene frequently involved in chromosomal translocations in leukaemia [114]. The partner protein forming the CBF complex, CBFβ, was also found in leukaemic translocations [92]. To obtain a better understanding of the processes

involved in leukaemia, it was essential to study the normal function of both genes. Mouse models were generated showing that Runx1 and CBFβ are implicated in the generation of HSCs during embryonic development [130,159,193].

Comparative analysis of the *Runx1* and *CBF*β knockout mouse models revealed nearly identical phenotypes. In both models the deficient embryos died between E12.5 and E13.5 due to lack of definitive hematopoiesis in fetal liver [130,159]. The embryos died displaying no or very few (respectively for *Runx1* -/- and *CBF*β -/-) hematopoietic progenitors in their fetal livers and yolk sacs. Detailed analysis of the Runx1 mouse models revealed that expression was essential for the presence of functional HSCs in the developing embryo [16]. Haploinsufficiency of *Runx1* results in a dramatic change in the temporal and spatial distribution of HSCs at embryonic stages. These embryos displayed an early appearance of HSCs in AGM and yolk sac. Thereafter, they disappear prematurely from the AGM region. The number of HSCs in the AGM of heterozygous embryos was reduced to 50 percent, whereas the homozygous knockout embryos completely lacked functional HSCs. These data show that emergence of HSCs in the AGM region is very sensitive to the levels of Runx1 protein.

HSCs emerging in the AGM region have been found in close association with the aortic endothelium. Hematopoietic clusters appear in chicken, mouse and human embryos on the ventral site of the dorsal aorta [34,51,183]. The appearance of hematopoietic clusters in the AGM coincides with the appearance of the first adult-repopulating HSCs. To determine the expression pattern of Runx1 during the emergence of HSCs, North and co-workers generated a mouse model expressing *LacZ* from the *Runx1* locus. They found *LacZ* expression in the prospective yolk sac blood island cells and the primitive erythroid precursors in the yolk sac. Later, at E8.5-9, Runx1 is expressed in the endothelial and mesenchymal cells of the PAS region (ventral), in the endothelial cells lining the vitelline artery, the fetal liver, the AGM region and in the endothelial cells of the yolk sac. Detailed analysis of the *LacZ*/*Runx1* expression in the AGM region showed the presence of *LacZ* positive cells in the hematopoietic clusters on the ventral site of the dorsal aorta, the endothelial cells of the aorta and in the mesenchyme underlying the aorta. Later it was shown that Runx1 expression marks long-term repopulating HSCs in the mid-gestation embryo [125]. Strikingly, analysis of the *Runx1* -/- embryos showed no hematopoietic clusters emerging from the dorsal aorta in the AGM. Overall, these data show that Runx1 plays an intrinsic role in HSCs during embryonic development within the different hematopoietic sites.

Since Runx1 is expressed in all major embryonic hematopoietic sites, plays a crucial role during embryonic development of the hematopoietic system, and is also highly associated with mutations and translocations in leukaemia it was expected that it would play a role in adult hematopoiesis. Expression of Runx1 in adult mice was studied through the generation and use of a Runx1-IRES-GFP mouse model [93]. *Runx1* expression was determined in myeloid, B- and T-cells and was found to be very weak in erythroid cells. Runx1 expression in T-cell subsets was found to be developmental stage-specific. North and co-workers supported these findings by analysing their Runx1-LacZ model [126]. They also showed that adult mouse HSCs from bone marrow all express Runx1. In depth analysis of *Runx1* +/- adult mice showed that the number of LTR-HSCs in these mice was reduced by 50% [176]. Surprisingly, this reduction was not extended to the level of hematopoietic progenitors. Instead, there was an increase

in progenitor cell levels in the adult mice. However, the regulation of differentiation towards mature blood cells was not affected. The levels of these cells were as in wild type mice. The only discrepancy detected was in the T-cell lineage and platelet formation, suggesting a more restricted role of Runx1 in these lineages. Ichikawa and co-workers used a conditional Runx1 knockout mouse model and addressed the functional affects of Runx1-deficiency on HSCs in these adult mice by transplantations [63]. Their results showed that *Runx1 -/-* bone marrow cells were unable to reconstitute T- and B-cells, whereas there was a normal reconstitution of mature neutrophils, monocytes and megakaryocytic populations. An absence of multi-lineage reconstitution with *Runx1 -/-* cells indicated a role for Runx1 in the differentiation of HSCs to mature blood cells in the adult.

1.4 Scope of this thesis

This introduction has provided an overview of our current knowledge of HSCs and hematopoietic progenitors during development and adult life. Their regulated emergence, expansion and differentiation via the microenvironment, cytokines, growth factors and transcription factors are complex processes. The interplay between all these regulators is still largely unknown and therefore intensive investigations are ongoing.

To gain more inside into how HSCs emergence and/or expansion is regulated I focused my thesis studies on an examination of the microenvironment and in particular on growth factors and transcription factors. In Chapter 2, I describe the effects of different FGFs on the hematopoietic supportive capacities of the AGM microenvironment. An *in vitro* equivalent of the AGM microenvironment, the UG26-1B6 stromal cell line, was used in these studies. We show for the first time that AGM-derived stromal cells express various *Fgf* and *Fgfr* transcripts and that stimulation of these cells by FGFs modulates the expression of mobilization factors. Upon exogenous addition of FGF1, 2 and 9 the supportive capacity of UG26-1B6 stromal cells for hematopoietic progenitors is enhanced. In contrast, FGF7 does not affect the supportive capacities of UG26-1B6 cells. The results suggest that FGF signalling plays a role in the AGM microenvironment. Furthermore, we showed that in adult bone marrow the effects of FGFs depend on the mouse strain, most likely due to differences in FGFR expression patterns on hematopoietic cells. Chapters 3 and 4 deal with the role of IL-1 as a hematopoietic cytokine in the mid-gestation embryo. Our studies suggested that within the AGM region, IL-1 signalling induces changes in expression of regulators of hematopoietic cell migration within the stroma, thereby influencing the proliferation, differentiation and mobilization of AGM HSCs. Also, IL-1 induces similar gene expression changes in the UG26-1B6 cell line, influencing the hematopoietic microenvironment *in vitro*. The microenvironment of other tissues has also been implicated in maintenance and/or expansion of HSCs and progenitors. One of the tissues important during embryonic development of the hematopoietic system is the fetal liver. In Chapter 4 it is reported that IL-1 in the fetal liver does not affect HSCs. Instead, IL-1 seems to act on hematopoietic progenitors directly or via the microenvironment. This indicates that IL-1 differentially regulates hematopoiesis within AGM and fetal liver. In Chapter 5 the

role of Runx1 is described. Although the crucial role of Runx1 within the HSCs was previously determined, the role of Runx1 within the supportive microenvironment remained unclear. In this study I determined that Runx1 is functional in the supportive capacities of the AGM microenvironment. This is independent of the genotype of the HSCs and progenitors, but is dependent on *Runx1* expression levels in the stroma. In Chapter 6 I attempt to integrate all the findings in a model/mechanism of regulation of HSCs and progenitors by the microenvironment.

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Chapter 2

Fibroblast Growth Factors regulate stromal cells and hematopoietic cells *in vitro*

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Abstract

The Fibroblast Growth Factor (FGF) signalling pathway is involved in angiogenesis, wound healing and hematopoiesis. Increasing evidence implicates a role of FGFs and FGFRs in the communication between the bone marrow microenvironment and hematopoietic progenitor cells (HPC) and stem cells (HSCs), affecting the growth and expansion of the latter. However, it is largely unknown what the nature of the interactions is and whether the effects of FGFs on hematopoietic cells are direct or indirect. Moreover, it has not been established whether the effects of FGFs differ between embryonic and adult type hematopoiesis. In this study we examined whether FGFs influence the HSC and HPC support in the embryonic aorta-gonad-mesonephros (AGM; the first tissue that generates adult-type HSC) microenvironment, using the well-characterized AGM stromal cell line UG26-1B6. This cell line has been shown to maintain adult HSC and HPC for up to 4 weeks during co-culture. Our results show that the support of HPC by the UG26-1B6 is enhanced in the presence of FGF1, FGF2 and FGF9. In contrast, FGF7 does not affect this support. We also compared the effects of FGFs on the bone marrow HSC supportive microenvironment in six mouse strains, as it is known that inbred mouse species differ in the size of the HSC pool as well as cycling activity. We show that long-term bone marrow cultures from 129Sv mice respond to FGFs best and are most likely mediated by the high-level expression of *Fgfr1* and *Fgfr3* in 129Sv cells with the phenotype of HSC. These results further add to our understanding of the role of FGF signalling in the cross-talk between hematopoietic cells and their microenvironment in the embryonic AGM, as well as the adult bone marrow microenvironment.

Introduction

The FGF super family consists of 22 members, all of which contain a highly conserved 120 amino acid core region [30]. Members of this family interact with heparin [28,30], which enhances their biological activities. FGFs act mainly extra-cellularly through four distinct tyrosine kinase receptors (FGFR1-4) [30]. Most of the FGFs are capable of binding and signalling through multiple receptors (e.g. Supplementary Figure 1 for FGFs used in this study), and some FGFs may act as nuclear transcription factors [13,28,30]. The FGF signalling pathway is highly complex and considered to play important roles in development [14], angiogenesis [13], hematopoiesis [11,12], wound healing and tumorigenesis [30]. FGFs and their receptors are also thought to be associated with the fate decisions of primitive cell subsets, as suggested by the temporal and spatial expression of specific FGFs and FGFRs during embryogenesis in specific cell types [36].

The two most studied FGFs are FGF1 (acidic FGF) and FGF2 (basic FGF). Both FGFs display an extensive sequence homology but have distinct expression patterns [30] with some overlap. In addition, they are involved in angiogenesis and act as mitogens for glial precursor cells, suggesting a redundancy in function [30]. Another interesting FGF family member, FGF9, is thought to act on nervous tissue, also displaying a mitogenic effect on different types of nerve cells. However, unlike FGF1 and FGF2, FGF9 is abundantly secreted and not able to bind to FGFR1 (Supplementary Figure 1) [28], which could explain why endothelial cells (which only express FGFR1) are not affected by FGF9. Strikingly, all three mentioned FGFs lack the leader sequences that would allow secretion of the factors through the classical polypeptide secretion pathway [30]. Several studies now provide evidence that the FGFs lacking a leader sequence are secreted through an ER- and Golgi-independent secretory pathway, similar to IL-1β [30,34].

Since FGF1 and FGF2 bind to all four known FGFRs and their isoforms (Supplementary Figure 1) [29], a role in developmental processes in many different cell types is suggested. However, involvement of FGFs in hematopoiesis is less well documented. Previous studies have shown that FGF2 promotes primitive erythropoiesis from embryonic cells [2,42]. More recently, de Haan and co-workers reported on the expansion of long-term repopulating HSCs in unseparated bone marrow cultures using FGF1 as the only stimulatory growth factor [11]. Because in the bone marrow the HSC population expresses FGFRs [11], signalling via other members of the FGF family might also be important in maintaining HSCs. Yeoh and co-workers [40] found a role for FGF2 in supporting HSC growth in culture. FGFs may also have a stimulatory effect on bone marrow stromal cells indirectly affecting hematopoietic cells [33,38]. This is supported by results showing a gradient of FGF4 expression within the bone marrow [1]. High levels of FGF4 have been shown to influence recruitment, proliferation and differentiation of HSCs [15,18,41].

At present, it is largely unknown whether FGFs affect the embryonic hematopoietic supportive microenvironment. During mouse development, the first adult-repopulating HSCs are autonomously generated in the aorta-gonads-mesonephros (AGM) region [21,23]. HSC numbers increase dramatically between embryonic day 10.5 (E10.5) and E12 in the AGM indicating that this is an uniquely sup-

portive microenvironment [16,21]. AGM-derived stromal cell lines have been shown to support HSC and hematopoietic progenitors during co-culture by providing a supportive microenvironment *in vitro* [25].

In the present study the effects of FGFs on the embryonic hematopoietic supportive microenvironment were examined. We report here the effects of FGFs on the supportive capacities of the AGM-derived stromal cell line UG26-1B6 and show that FGF1, 2 and 9, but not FGF7, promote the *in vitro* growth of immature bone marrow hematopoietic progenitors. Also, since it has been shown that inbred strains of mouse differ in the cycling activities of HSCs and the size of the HSC pool [9], we examined the strain specific effects of FGFs on BM HSC.

Material and Methods

Mouse strains, cells and cell lines

Mouse strains (C57BL/6, (C57BL10xCBA)F_1, 129Sv, FVB.n, Balb/cAnCrLBr, CBA/Jico and C3H HeJ) used in these studies were maintained according to institutional guidelines, with free access to food and water. Animal procedures were carried out in compliance with Standards for Humane Care and Use of Laboratory Animals. Femurs and tibias from male mice were collected and crushed using mortar and pestle. Single cells were collected in PBS (Gibco Inc.), 10% heat-inactivated filtered FCS (GibcoBRL) and Pen/Strep (GibcoBRL) (PBS/FCS/PS) and washed twice.

The UG26-1B6 cell line was grown on pre-coated (0.1% gelatin) dishes in LTC-SM medium (50% M5300 (StemCell Technologies Vancouver, Canada), 15% heat-inactivated filtered FCS (GibcoBRL), 35% alpha-MEM (GibcoBRL), 10 μm β-mercaptoethanol (Sigma) and Pen/Strep (GibcoBRL)) at 33°C in a humidified chamber under 5% CO_2.

FACS analysis

Cells were harvested and washed in PBS/FCS/PS twice before the labelling for 30 minutes on ice. Lineage labelling (Lin) was performed using the Mouse Lineage Panel (BD Pharmingen) containing biotin-labelled CD3e, CD45R/B220, Ly-6G/Gr-1, CD11b/Mac-1 and Ter-119/Ly-76 antibodies. The Mouse Lineage Panel was visualized by staining with Streptavidin-PerCP-Cy5.5 (BD Pharmingen). Other labels included phycoerythrin-conjugated anti-Sca-1 (Ly-6A/E) (BD Pharmingen) and allophycocyanin-conjugated anti-c-kit (CD117) (BD Pharmingen) antibodies. Dead cells were eliminated by negatively selecting for Hoechst 33258 (1μg/ml) (Molecular Probes) labelled cells. Flow cytometric analysis was performed using a FACSAria and Diva software (BD).

Enrichment of bone marrow hematopoietic cells for co-culture

The discontinuous ficoll gradient from E. Schneider [35] was adapted for murine bone marrow cells and performed using a 30% w/w solution (31.5 gram Ficoll-400 (Pharmacia) dissolved in 100 ml 0.1 M sodium phosphate solution pH=7.4). This 30% w/w solution was diluted into 19% w/w and 22% w/w with the sodium phosphate buffer. The discontinuous gradient was layered as follows: 3.5 ml 22% w/w

Ficoll-400, 2.5 ml 19% w/w Ficoll-400, 1.5 ml containing 2-3x10^8 cells in PBS/FCS/PS in Beckman Pollyallomer (14x89 mm) tubes. Ultracentrifugation was performed in a pre-cooled Beckman Optima™L using a swing-out SW40Ti rotor with buckets at 13.800 rpm, 4°C for 35 minutes with slow acceleration and no break. Low-density bone marrow cells layered between 1.069 g/ml (19% w/w) and 1.075 g/ml (22% w/w) were collected. Cells were then washed twice in PBS/FCS/PS before labelling with phycoerythrin-conjugated anti-CD31 (PECAM1) (BD Pharmingen), isothyocyanate-conjugated anti-Ly-6C (ER-MP20) (BD Pharmingen) and allophycocyanin-conjugated anti-c-kit (CD117) (BD Pharmingen) antibodies for 30 minutes on ice. CD31^{med} Ly-6C^{-} c-kit^{high} cells were sorted on the FACSAria (Becton Dickinson). Dead cells were eliminated by negatively selecting for Hoechst 33258 (Molecular Probes) as described before.

In vitro hematopoietic cultures

For the colony-forming-unit in culture (CFU-C) assay, cells were washed in PBS/FCS/PS. Cells were seeded in triplicates at concentrations varying between 0.5x10^4 and 2x10^4 cells per plate (co-cultures with unfractionated bone marrow cells) or an equivalent of 50-200 co-cultured cells (co-cultures with enriched bone marrow cells) in methylcellulose medium (Methocult™ M3434; StemCell Technologies Inc.) supplemented with stem cell factor (SCF) (50 ng/ml), IL-3 (10 ng/ml), IL-6 (10 ng/ml) and Epo (3 U/ml). All cultures were incubated at 37°C in a humidified chamber under 5% CO_2. Colony-forming unit-granulocyte (CFU-G), colony-forming unit-macrophage (CFU-M) and colony-forming unit-granulocyte macrophage (CFU-GM) were scored with an inverted microscope at day 7 of the culture.

For the co-culture, the UG26-1B6 stromal cell line was seeded in 0.1% gelatin pre-coated 25 cm^2 flasks, grown to confluency and irradiated (20 Gy). Either 10^6 bone marrow cells or 2000 enriched bone marrow cells were seeded onto the irradiated UG26-1B6 stromal layer using LTC-SM medium containing heparin (10 μg/ml; Organon Technika) and/or hu-FGF1, hu-FGF2, hu-FGF7 and hu-FGF9 (all from R&D systems) at final concentration of 10 ng/ml. The cultures were maintained for 1 week at 33°C, 5% CO_2. After 4 days, the cultures were supplemented with fresh growth factors and heparin. For experimental set up see Figure 2A.

For long-term bone marrow cultures (LTBMCs), cells were suspended in StemSpan™ SF Expansion Medium (StemCell Technologies Inc.) containing Pen/Strep (GibcoBRL), 10 μm β-mercaptoethanol (Sigma) and heparin (10 μg/ml; Organon Technika). Cells (2x10^7) were seeded in pre-coated (0.1% gelatin) 25 cm^2 flasks (Corning-Costar Inc.). Hu-FGF1, hu-FGF2 and hu-FGF9 (R&D systems) at 10 ng/ml were added accordingly and the cultures were maintained for 5 weeks at 33°C, 5%CO_2. Each week, half of the medium was removed and non-adherent cells were recovered. These cells were added back to the cultures, along with fresh medium containing FGFs (20 ng/ml). For experimental set up see Figure 2B.

RNA isolation, cDNA synthesis and RT-PCR analysis

Total RNA was isolated with TRIZOL (Invitrogen) according to the manufacturer's protocol and DNAse treated with RQ1 RNase free DNAse (Promega). For cDNA synthesis 0.5 – 2 μg of total RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen/Life Technologies) according to

manufacturer's instructions. PCR reactions were performed in 50 µl containing 1 unit of Amplitaq (PerkinElmer) and the provided buffer, 100 ng of reverse and forward primer, 1 mM dNTP-mix and 1-2 µl of cDNA. *Fgfr4* and *Fgf1* RT-PCRs also contained DMSO. The PCR cycles were as follows: 5 minutes 92°C, 30-40 cycles (1 minute 92°C, 1 minute 58°C, 1 minute 72°C) and 7 minutes at 72°C. For primer details see Supplementary Table 1. The PCR products were run on 1-2% agarose/1x TBE gels with etidium bromide, scanned using a Typhoon scanner (Molecular Dynamics) and analysed with ImageQuant Software.

Total RNA was isolated from LSK cells using TRIZOL (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol and cDNA was synthesized using a SUPERSCRIPT system (Gibco BRL). The cDNA was amplified with ExTaq polymerase (Takara Shuzo, Osaka, Japan). Real-time RT-PCR was performed using an iCycler (Bio-Rad, Hercules, CA) and qPCR Core kit for SYBR Green (Eurogentec, Seraing, Belgium) with the following parameters: 3 minutes 95°C, 50 cycles (15 sec 95°C, 20 sec 61.5°C, 40 sec 72°C). The cDNA quantities were normalized to the amount of *Hprt* cDNA. For primer details see Supplementary Table 1.

Results

Expression of FGFs and FGFRs in the UG26-1B6 stromal cell line

Previously it was shown that a stromal microenvironment is necessary to support bone marrow and embryonic HSC, LTC-IC and human cord blood HSC *in vitro*[17,25]. One of the most potent stromal cell lines providing a supportive microenvironment *in vitro* is UG26-1B6 derived from the AGM microenvironment. UG26-1B6 supports all of the above mentioned HSCs species [17,25]. Recently, Oostendorp and co-workers [26] showed that *Fgf7* and *Fgf9* RNA transcripts are elevated in AGM-derived cell lines supportive for HSCs (UG26-1B6 and EL08-1D2) as compared to non-supportive cell lines (UG15-1B7, EL28-1B2, AM20-1B4 and AM30-3F4) using a macro-array approach. To further confirm these results we examined the UG26-1B6 cell line for expression of *Fgfs* and *Fgfrs* by RT-PCR analysis. Although we detected high expression of *Fgf7*, *Fgf9* expression was not found (Figure 1A). We also found low expression of *Fgf2* and no expression of *Fgf1* (Figure 1A). Regarding receptor expression, both *Fgfr1* and *Fgfr2* were highly expressed, while *Fgfr3* and *Fgfr4* transcripts were barely or not detectable (Figure 1A).

Cytokines and growth factors are known to regulate expression of numerous target genes by activating downstream pathways through their receptors. To examine whether FGF signalling affects the expression of their own receptors (auto regulation), FGF1, 2, 7 and 9 were added to cultures of UG26-1B6 cells (along with heparin) and RT-PCR analysis was performed after 2 days. Expression analysis (Figure 1B) showed an increase in *Fgfr1* and *Fgfr3* when FGF7 was added to the cultures. Exogenously added FGF2 increased *Fgfr1* and decreased *Fgfr2* expression, while FGF9 increased *Fgfr1* and *Fgfr3* expression and slightly decreased *Fgfr2* expression. In contrast, FGF1 resulted in decreased *Fgfr2* expression and appeared to have negligible affects on the expression of the other receptors. Thus,

FGFs appear to affect the expression of their receptors, which might contribute to functional changes of the UG26-1B6 cells.

Effects of FGFs on LSK cell support in bone marrow co-cultures with UG26-1B6 stromal cells

To test the effect of FGFs on the hematopoietic supportive capacity of the UG26-1B6 stromal cell line, unfractionated murine bone marrow cells (C57BL6) were co-cultured with this stromal cell line and in the presence of either FGF1, FGF2, FGF7, FGF9 or control medium (Figure 2A). All groups contained heparin. After 1 week, the total number of viable cells per flask was assessed. Both the control cultures (no growth factors added) and co-cultures supplemented with FGFs displayed a slight increase in total number of cells as compared to the inoculum (Table 1).

To analyse whether the presence of FGFs affects the support of bone marrow HSCs, we analysed the co-cultures with UG26-1B6 in the presence or absence of FGFs for the presence of $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK) cells. The number of LSK cells was examined one week after initiation of the co-cultures (Figure 3A). Co-cultures supplemented with either FGF1, FGF2 or FGF9 showed maintenance and growth of input number of LSK cells and were about 2-fold increased as compared to the no growth factor control. Co-cultures supplemented with FGF7 showed similar support for LSK cells as the no growth factor control. These results suggest that FGF1, FGF2 and FGF9 expand LSK cells during 1-week co-culture on the UG26-1B6 stromal cell line, whereas FGF7 does not.

FGFs affect the growth of hematopoietic progenitors during co-culture on UG26-1B6 stromal cells

In addition to our study of FGF effects on LSK cells we also functionally assessed the support of hematopoietic progenitors during co-culture using the CFU-C assay. We analysed granulocyte (CFU-G), macrophage (CFU-M) progenitors and granulocyte-macrophage (CFU-GM) progenitors present in the co-cultures of UG26-1B6 stromal cells seeded with 10^6 whole bone marrow cells. In general, none of the co-cultures where able to maintain the input numbers of CFU-C (10567 ± 3454 CFU-C/10^6 BMCs; data not shown). Co-cultures supplemented with the different FGFs supported only 12-21% of input CFU-C and the no growth factor control maintained only 14% of the input CFU-C numbers (data not shown). Addition of FGF1, 2 and 9 to the co-cultures resulted in a slight increase in the number of CFU-M progenitors (Figure 3B) compared to the no growth factor control (1.3-, 1.5- and 1.3-fold respectively). Although less pronounced, CFU-G and CFU-GM were also slightly increased upon addition of FGF1, 2 and 9 compared to no growth factor control (ranging between 1.3 and 1.7-fold increase) (Figure 3B). In contrast, co-cultures supplemented with FGF7 showed little to no change in CFU-G, CFU-M and CFU-GM progenitors compared to no growth factor. Thus, FGF1, 2 and 9, but not FGF7, stimulate hematopoietic cell growth in co-cultures with UG26-1B6 stroma.

Whole bone marrow contains not only hematopoietic cells, but also many cells of the stromal micro-environment that could affect the co-culture system. Thus in further studies we enriched for immature hematopoietic progenitors prior to co-culture. Using flow cytometric sorting, CFU-Cs were enriched 20-

fold and LTR-HSCs ~300-1000 fold ([25]; data not shown). Two thousand sorted bone marrow cells were co-cultured with UG26-1B6 cells in the presence or absence of FGFs in multi-well plates. After 1 week of co-culture cells were harvested and we assessed the support of functional hematopoietic progenitors by the CFU-C assay. In contrast to the results with unfractionated bone marrow, all co-cultures seeded with sorted bone marrow progenitors showed an increase in the total number of CFU-C as compared to input CFU-C numbers (430 ± 79 per well; data not shown). In the no growth factor control, CFU-C were increased 2.3-fold (1007 ± 118 per well) and in the co-cultures supplemented with FGF1, 2, 7 and 9 CFU-C numbers were increased by factors of 4.8, 5.5, 2.1 and 3.6 respectively, compared to input CFU-C numbers. As shown in Figure 3C, when compared to the no growth factor control co-culture, the sorted bone marrow cells supplemented with FGFs yielded high numbers of CFU-G, CFU-M and CFU-GM. FGF1, 2 and 9 provided the best support (3.8-, 6.4- and 6.7-fold increase of CFU-GM). Co-cultures supplemented with FGF7 supported mature hematopoietic progenitors (CFU-G and CFU-M) similar to the no growth factor control, whereas they increase the number of CFU-GM (113 ± 22 vs. 33 ± 7 per well). The progenitor cell increases observed in co-cultures established with sorted bone marrow cells suggest that the FGFs stimulate the production of CFU-Cs from HSCs and immature progenitors.

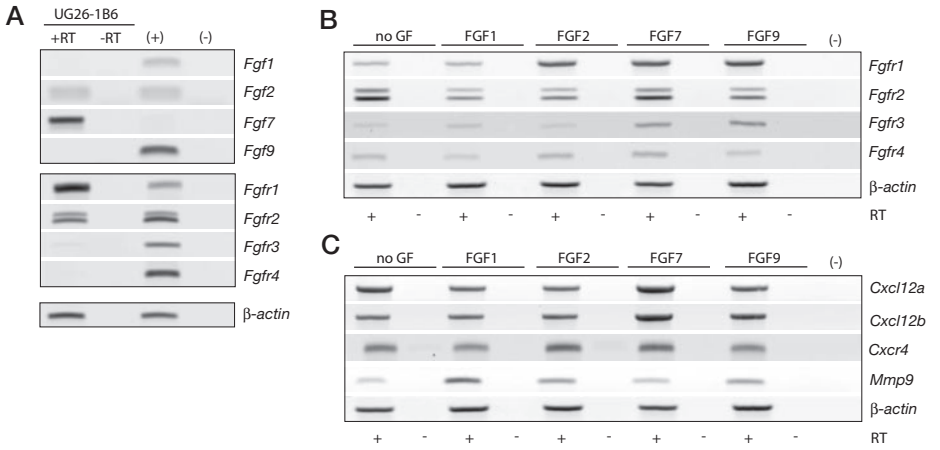

Figure 1. Expression of *Fgfs*, *Fgfrs* and components of the hematopoietic microenvironment by the UG26-1B6 stromal cell line

RT-PCR analysis was performed on UG26-1B6 (irradiated) stromal cells grown for 2 days to examine **(A)** *Fgf* and *Fgfr* expression, and UG26-1B6 cells (irradiated) grown for 2 days with or without FGFs (10 ng/ml) in medium containing heparin to examine **(B)** *Fgfr* expression and **(C)** chemokines *Cxcl12a* and *Cxcl12b*, the Cxcl12 receptor *Cxcr4* and the extracellular matrix protein *Mmp9*. β-*actin* expression was used as normalization control. RT-PCR analysis was performed on samples treated with (+RT) and without (–RT) reverse transcriptase. (+) = positive control sample and (-) = negative control sample.

FGFs influence UG26-1B6 morphology and gene expression

The morphology of UG26-1B6 cells in co-cultures with and without FGFs was examined. Control co-cultures contained a well-developed confluent layer of UG26-1B6 stromal cells and areas of hematopoietic activity, both on top of the stroma (refractive cells, arrowheads) and underneath (phase-contrast dark round cells often organized as cobblestone areas, arrows) (Figure 4A). Co-cultures containing FGF7 (Figure 4D) had a similar appearance to the control co-cultures. In contrast, co-cultures supplemented with FGF1, 2 and 9 differed in their morphology (Figure 4B, C, E). In these co-cultures, many UG26-1B6 cells were elongated, with thin processes emanating over a long distance from the slightly rounded cell body. As a result, the stromal layer incompletely covered the bottom of

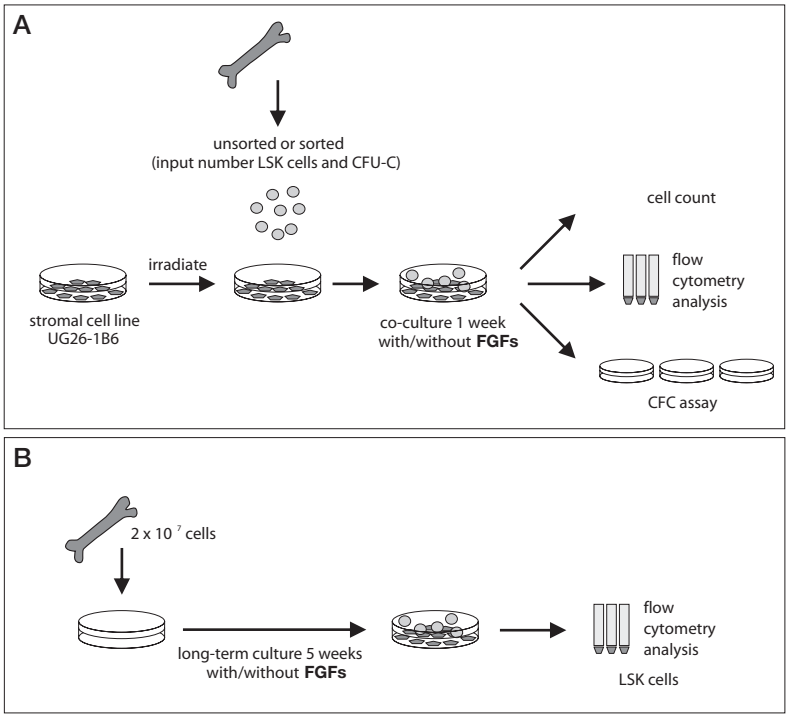

Figure 2. Experimental procedure

(A) Co-culture strategy used to study the hematopoietic supportive capacities of UG26-1B6 stromal cell line and the effects of exogenously added FGFs. UG26-1B6 stromal cells were seeded into 6-well plates or 25 cm^2 flasks, grown to confluency and irradiated (20 Gy). Cultures were seeded with the (enriched) bone marrow populations, of which input numbers of LSK cells and CFU-Cs were determined prior to co-culture, and grown for 1 week with or without FGFs (all cultures contained heparin). Subsequently, HSC phenotypic profiling and the presence of hematopoietic progenitors by CFU-C assay were performed. **(B)** One-step long-term bone marrow cultures (LTBMCs) used to study the effects of exogenously added FGFs. Twenty million unfractionated bone marrow cells of 6 different mouse strains were seeded into 25 cm^2 flasks in medium with or without FGFs (all cultures contained heparin). After 5 weeks the cultures were analysed using flow cytometry analysis to determine the number of LSK cells.

the wells. Hematopoietic activity was less prominent than in control co-cultures, with fewer refractive and phase-dark cells. These descriptive results, together with the results of the CFU-C assays, suggest a correlation between the morphological changes in the supportive stromal cells and hematopoietic (progenitor) cell growth observed in the presence of the various FGFs.

We next examined whether FGFs affect the expression of molecules which might be involved in the communication between hematopoietic cells and cells of the microenvironment. RT-PCR analysis was performed on RNA from UG26-1B6 cells grown for 2 days in the presence of exogenously added FGF1, 2, 7 and 9 plus heparin (Figure 1C). Analysis of mobilization factors *Cxcl12a* (SDF-1α) and *Cxcl12b* (SDF-1β) and their receptor *Cxcr4* showed no or little change in expression levels in the presence of FGF1, 2 or 9. However, FGF7 upregulated the expression of *Cxcl12b*. Increased *Mmp9* expression was observed after treatment with FGF1, 2 and 9 but not FGF7. Thus, FGFs differentially influence the morphology and expression of some of the components of the hematopoietic microenvironment.

Effects of FGFs on LSK cells in long-term bone marrow cultures are mouse strain dependent

Femoral cellularity differences between inbred mouse strains are thought to reflect the overall content of HSC [7]. C57BL/6J mice display a high cellularity (21.7 x 10^6 cells/femur) whereas C3H/HeJ mice have a lower cellularity (12.6 x 10^6 cells/femur) [7]. Moreover, strain-related differences in HSC numbers have been shown to correlate with cell cycle characteristics and aging [7,10]. Since FGFs influence the *in vitro* growth of hematopoietic progenitors, we examined the effects of FGF1, 2 and 9 in LTBMCs derived from 6 inbred mouse strains. After 5 weeks of culture in the presence or absence of these factors, we found striking differences in the growth of immature hematopoietic cells (defined by the flow cytometric LSK profile) from the different strains (Table 2). In the absence of FGFs, C3H/HeJ bone marrow supported the lowest number of LSK cells (0.28 x 10^3 cells) and 129Sv LTBMC the highest LSK cell number. FVB, Balb/c, CBA and C57BL/6 LTBMC were more similar in output and yielded intermediate numbers of LSK cells.

Table 1. Bone marrow cell growth in FGF-supplemented co-cultures with the AGM stromal cell line UG26-1B6

Growth factors added to the cultures	Absolute number of cells (x 10^6) per flask (average ± 2SEM)
no GF	1.68 ± 0.34
FGF1	1.56 ± 0.25
FGF2	1.61 ± 0.27
FGF7	1.39 ± 0.31
FGF9	1.46 ± 0.49

One million bone marrow cells were seeded in culture flasks containing a pre-established irradiated monolayer of UG26-1B6 stromal cells. The co-cultures were supplemented with FGF1, 2, 7 and 9 except for the no GF control. All co-cultures contained heparin (10 µg/ml) during the total culture period. Viable cell number per flask was analysed after 1 week for each condition by automatic cell counter (n=4). GF = growth factor.

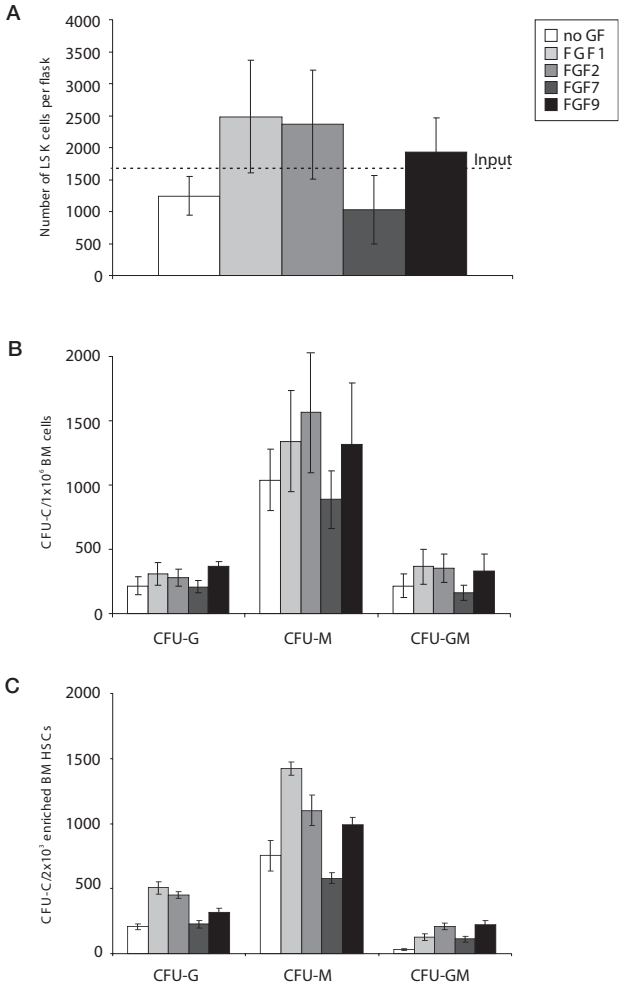

Figure 3. Hematopoietic progenitor support provided by FGFs in UG26-1B6 co-cultures

Growth of hematopoietic progenitors in FGF supplemented co-cultures was analysed. **(A)** $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ (LSK) cell numbers per flask were analysed after 1 week of co-culture of whole bone marrow cells with a monolayer of irradiated UG26-1B6 cells in the presence or absence of FGFs (and heparin). Absolute numbers of LSK cells were determined for each condition (n=4). On average the input number of LSK cells seeded per flask was 1681 ± 117 cells. **(B)** CFU-G, CFU-M and CFU-GM per 10^6 bone marrow cells were analysed after 1 week of co-culture of whole bone marrow cells with a monolayer of irradiated UG26-1B6 cells in the presence or absence of FGFs (and heparin). Results of 4 experiments are displayed as CFU-Cs ± 2SEM per 10^6 bone marrow cells. The number of initiated CFU-Cs per 10^6 BMCs was 1600 ± 552 CFU-G; 6125 ± 1885 CFU-M; 2133 ± 829 CFU-GM. **(C)** CFU-G, CFU-M and CFU-GM per 2 x 10^3 light density $CD31^{med}$ $c\text{-}kit^{hi}$ $Ly\text{-}6C^{-}$ bone marrow cells were analysed after 1 week of co-culture of enriched bone marrow cells with a monolayer of irradiated UG26-1B6 cells in the presence or absence of FGFs (and heparin). Results of 2 experiments are displayed as CFU-Cs ± 2SEM. The number of initiated CFU-Cs per 2 x 10^3 $CD31^{med}$ $c\text{-}kit^{hi}$ $Ly\text{-}6C^{-}$ LD BMCs was 33 ± 4 CFU-G; 350 ± 15 CFU-M; 240 ± 92 CFU-GM. GF = growth factor.

As previously described for co-cultures with the UG26-1B6, FGFs affected the morphology of LTBMCs. Many stromal cells in the cultures supplemented with FGF1, 2 and 9 were more elongated and less flattened, resulting in a stromal layer with incomplete confluency. We compared the LSK output from LTBMCs of the different strains supplemented with FGF1, 2 or 9. As shown in Table 2, LSK cell growth in LTBMCs supplemented with FGF1 was increased in cultures from 129 (5.1-fold), FVB (8.1-fold) and Balb/c (2.2-fold) mice over no growth factor control LTBMCs. The CBA and the C57BL LTBMCs showed a decrease in LSK cell growth (5.0- and 14.4-fold decrease compared to no growth factor LTBMCs, respectively). The C3H mouse strain was able to maintain same low levels of LSK cells as without growth factors. The same trend was seen in the LTBMCs supplemented with FGF2, with increased numbers of LSK cells in 129, FVB and Balb/c (1.4-, 1.8- and 2.2-fold, respectively), decreased numbers in CBA (2.3-fold) and C57BL (10.1-fold) and no change in C3H LTBMCs. Addition of FGF9 to the LTBMCs elicited an increase in LSK cell numbers (2.7-fold) only in the 129 cultures. Hence, the effects of FGFs on the growth of LSK cells in LTBMCs are dependent on the genetic background.

To test whether these differences could be due to *Fgfr* expression by LSK cells, we performed real-time RT-PCR on sorted LSK cells from two of the most disparate strains, 129 and C57BL. The overall femur cellularity of 129 mice was less than in C57BL mice ($3.4 \pm 0.2 \times 10^7$ cells/femur vs. $6.6 \pm 0.9 \times 10^7$ cells/femur). As shown in Figure 5, *Fgfr1* and *Fgfr3* expression were respectively 2-fold and 5-fold higher on the LSK cells of 129 bone marrow as compared to C57BL. *Fgfr2* was not detected in either strain and *Fgfr4* was expressed on LSK cells only by the 129 strain (data not shown). These data suggest that strain-specific responsiveness to FGFs is dependent, at least partly, upon *Fgfr* expression levels.

Discussion and conclusion

In this study we investigated the effects of FGFs on adult stem and progenitor cells as well as on the hematopoietic microenvironment. We have shown that the UG26-1B6 stromal cell line expresses a variety of *Fgfs* and *Fgfrs* and that addition of FGFs modulates the expression of *Fgfrs* and mobilization regulators. In addition, exogenously added FGF1, FGF2 and FGF9, but not FGF7, stimulates the supportive capacity of the UG26-1B6 embryonic stromal cell line for the growth of CFU-C and LSK cells *in vitro*. The positive effect of these three FGFs appears to be mouse strain-specific and most likely due to differences in *Fgfr* expression patterns on immature hematopoietic cells.

FGFs affects are mouse strain-specific

There is accumulating evidence for mouse strain-specific differences in bone density, life span and body weight [3]. Also bone marrow HSCs vary in their frequency and proliferation status between mouse strains [7,8]. Since maintenance and proliferation of HSCs is largely dependent on the cross-talk between HSCs and their microenvironment [18,41] LTBMCs are a powerful tool for studying strain-specific differences, as it allows one to study the hematopoietic and stromal compartment *in vitro* simultaneously. The stromal layer is formed within 7-10 days of culture and it supports hematopoietic activity for

many weeks. Indeed, we found strain-specific differences in LSK growth in LTBMCs (Table 2). Further we observed that addition of FGFs from the beginning of these cultures resulted in altered stromal cell morphology and in a discontinuous stromal layer that could affect of the supportive capacity of the LTBMCs. Despite the affected morphology FGF1 and FGF2 still increased the LSK output in the LTBMCs of several mouse strains (Table 2). However, FGF9 positively affected the number of LSK cells in LTBMCs of only the 129-mouse strain. Overall, the femur cellularity of 129 mice is less than in C57BL6 mice and is thought to reflect the overall content of HSC [7]. We found that 129 mice contained on average 911 ± 99 LSK cells/10^6 BMCs whereas 1681 ± 117 LSK cells/10^6 BMCs were found in the C57BL/6 strain (unpublished data). This data, together with the difference in *Fgfr* expression levels in

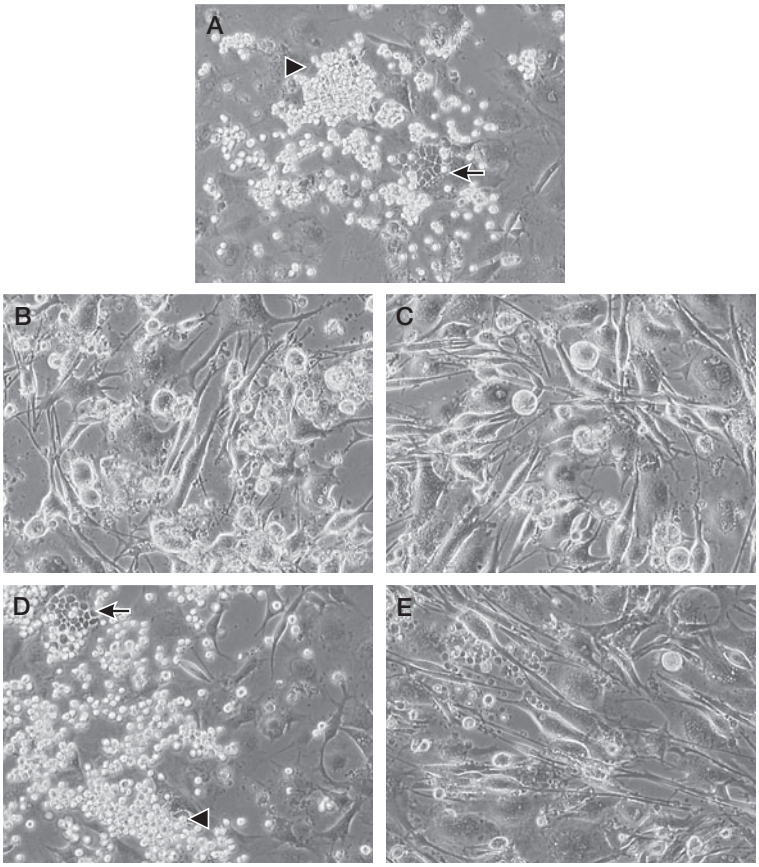

Figure 4. Morphology of hematopoietic co-cultures supplemented with FGFs
Light density CD31^{med} c-kit^{hi} Ly-6C^{-} bone marrow cells (2 x 10^3) were seeded on a confluent monolayer of irradiated UG26-1B6 stromal cells and cultured for 7 days in the absence or presence of FGFs (10 ng/ml). All co-cultures contained heparin (10 µg/ml). Co-cultures containing **(A)** no growth factor; **(B)** FGF1; **(C)** FGF2; **(D)** FGF7; and **(E)** FGF9. Black arrows indicate cobblestone-areas (phase dark) and black arrowheads indicate hematopoietic-like colonies on top (refractive).

the LSK cells (Figure 5), indicate important strain-specific hematopoietic cell regulatory differences that are at least in part due to the FGF signalling pathway.

Influences of FGFs on downstream target genes

In our study we confirmed the finding of Oostendorp et al. that the AGM (subregion)-derived stromal cell line UG26-1B6 expresses high levels of *Fgf7* [26]. We showed for the first time the presence of *Fgfr1* and *Fgfr2* in this stromal cell line suggesting that FGF signalling in AGM stromal cells could play a role in the maintenance and/or expansion of HSCs. In fact, FGFs modulate the expression of their receptors (Figure 1B) and some other mobilization regulators (Figure 1C) in the UG26-1B6 cells.

The FGF (1, 2, 9) stimulated increase in CFU-C and LSK cells in our co-culture system (Figure 3B, C) may be linked to the fact that these FGFs modulate the expression of matrix metalloproteinase 9 (*Mmp9*). Indeed, we found upregulated expression of *Mmp9* in UG26-1B6 cells cultured in the presence of FGF1, 2 and 9 (Figure 1C). Mmp9 degrades collagens of the extracellular matrix, resulting in the mobilization of HSC/HPC by detaching them from the stromal compartment [27,32]. Furthermore, by cleaving the extracellular matrix temporarily stored growth factors and cytokines could be released. One of the pathways used by FGFRs to transmit their signals is the Ras/MAPK pathway [4], which recently has been implicated to increase Mmp9 secretion when FGF2 was added [5,19,24]. Therefore, increased levels of *Mmp9* transcripts in the UG26-1B6 cells could induce proliferation and differentiation of immature hematopoietic cells leading to their detachment from the stromal cells. When HSCs and HPCs are anchored to the stromal niche cells they are able to maintain their immature state.

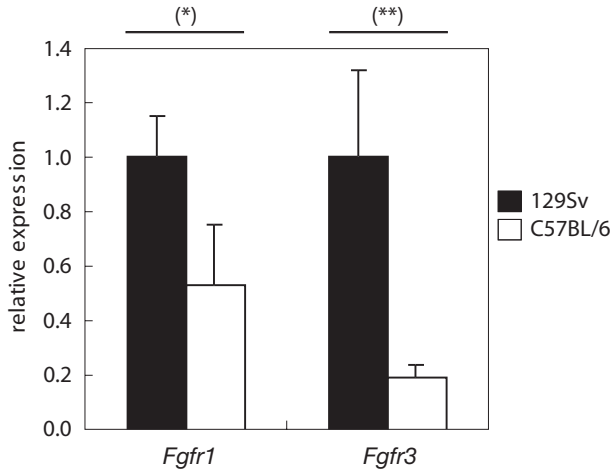

Figure 5. *Fgfr1* and *Fgfr3* expression analysis of bone marrow LSK cells from 129Sv and C57BL/6 mouse strains

Real-time RT-PCR analysis of *Fgfr1* and *Fgfr3* expression by LSK cells was performed. The bar graph shows the relative expression levels of *Fgfrs* by the LSK populations of 129Sv and C57BL/6 mice (n=3). The expression levels are normalized against *Hprt* expression. (*) P = 0.04 and (**) P = 0.01.

Table 2. Effects of FGFs on the long-term *in vitro* growth of LSK cells from various mouse strains

Mouse strain	Number of LSK cells (x 10^3) per flask (fold increase or decrease)						
	No GF	FGF1		FGF2		FGF9	
129	18.90	95.74	(+5.1)	26.43	(+1.4)	50.21	(+2.7)
FVB	4.13	33.49	(+8.1)	7.10	(+1.8)	0.78	(-5.3)
Balb/c	2.07	4.48	(+2.2)	4.63	(+2.2)	0.47	(-4.4)
CBA	1.98	0.40	(-5.0)	0.87	(-2.3)	0.35	(-5.7)
C57BL	0.81	0.06	(-14.4)	0.08	(-10.1)	0.11	(-7.4)
C3H	0.28	0.29	(0)	0.26	(0)	0.15	(-1.9)

Cultures of bone marrow cells (2 x 10^7 cells per 25 cm^2 flask) from a panel of mouse strains were grown for 5 weeks and analysed for LSK cell number by flow cytometry. Cultures were supplemented with FGF1, 2, 9 or no growth factor. All cultures contained heparin (10 µg/ml). The table displays the absolute number of LSK cells per flask. GF = growth factor

A key-player in the homing of HSCs and HPCs to the niches is the Cxcl12/Cxcr4-signaling pathway. Cxcl12 is a chemokine, which, when present at high levels, attracts HSCs and HPCs expressing Cxcr4. Cells comprising the adult bone marrow niche are known to express Cxcl12 at high levels creating a gradient (between the niches and the blood vessels) [6,15,37,41]. Upon stimulation with FGF7 we found that the UG26-1B6 stromal cells show elevated levels of *Cxcl12b* (one of the two isoforms of Cxcl12). This suggests that the HSC and/or HPC in the co-culture may be increased in their attraction to the stromal cells. Interestingly, Mmp9 acts on Cxcl12 neutralizing its function as attractant [20]. Since FGF7 addition does not increase *Mmp9* transcripts in UG26-1B6 cells homing of HSC and/or HPC to the stromal cells might be improved. This is supported by the fact that FGF7 stimulation did not increase the number of CFU-G and CFU-M and maintained similar numbers of LSK cells in co-culture, compared the no growth factor. Using the enriched bone marrow HSCs in the co-cultures we observed an increase in the CFU-GM numbers, whereas using whole bone marrow as inoculum we did not. This discrepancy could be due to the presence in whole bone marrow of other cell types like stromal cells, which can express chemoattractants and therefore influence the homing of immature hematopoietic cells. Using an enriched HSC population in co-cultures eliminates this "confusion in homing". Clearly, the FGF mediated modulation in the expression of mobilization/homing factors by UG26-1B6 should be studied further and should also address whether FGFs modulate cytokines and growth factor production of the UG26-1B6 stromal cells. To determine whether UG26-1B6 stromal cells are influenced by the FGFs in the maintenance and/or expansion of functional HSCs, *in vivo* long-term transplantation assays should be performed. This would be particularly interesting using the co-cultures supplemented with FGF7, since upon increased homing (induced by increased levels of *Cxcl12b* transcripts) HSCs could be allowed to maintain their immature state.

Since there are 22 FGF-family members displaying different affinities to their 4 FGFRs which are expressed on hematopoietic cells as well as cells of the adult and embryonic microenvironment, the regulatory role of FGF signalling in hematopoiesis is likely to be very complex. Adding to this complexity, FGF signalling can interact with other pathways that play a role in the hematopoietic

system, e.g. with the BMP-4 pathway [39]. Our results add to the understanding of FGF signalling in the cross-talk between hematopoietic cells and cells of the embryonic and adult microenvironments. As FGF signalling is also implicated in the development of osteoblasts [22] and the formation of blood vessels [31] (both processes linked to the hematopoietic system) there is an even larger role for FGFs that requires further studies.

Acknowledgements

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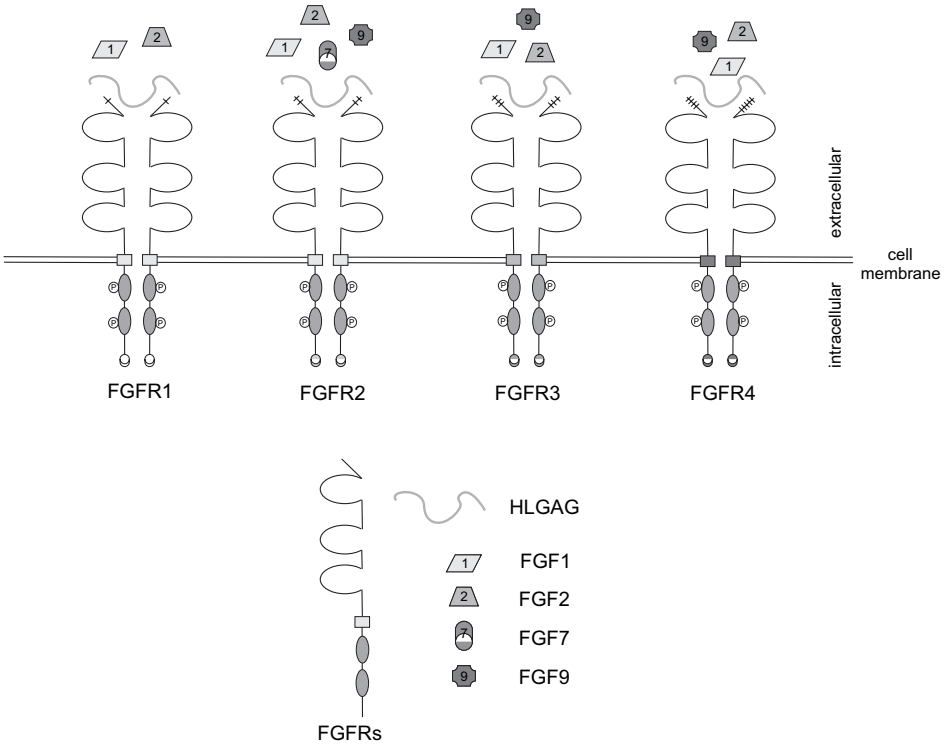

Supplementary Figure 1. Binding of FGFs to their receptors
FGFs have different binding affinities to their FGFRs. By interacting with heparin, the biological activity of FGF signalling is enhanced and the binding between FGFs and FGFRs is stabilized. FGF1 and FGF2 are able to bind all four FGFRs. FGF9 can bind to all FGFRs except to FGFR1, whereas FGF7 can only bind to FGFR2

Supplementary Table 1. RT-PCR primer sequences

Primer name	Primer sequence (5'-3')	Product size (bp)	Accession number
β-*actin* for	CCTGAACCCTAAGGCCAACCG	397	X03672
β-*actin* rev	GCTCATAGCTCTTCTCCAGGG		
Fgf1 for	GACCGAGAGGTTCAACCTGC	131	NM_010197
Fgf1 rev	CCTTCTGGAAACCTCAGGCC		
Fgf2 for	GCTATGAAGGAAGATGGACG	217	NM_008006
Fgf2 rev	CAGTGCCACATACCAACTGG		
Fgf7 for	GGATACTGACACGGATCCTG	465	NM_008008
Fgf7 rev	CTGTGTGTCCATTTAGCTGATGC		
Fgf9 for	GCAGCTGTACTGCAGGACTG	282	NM_013518
Fgf9 rev	CTCCTTCCGGTGTCCACATG		
Fgfr1 for	ACCGTGTGACCAAAGTGGCC	509	NM_010206
Fgfr1 rev	CGGTCAAACAACGCCTCAGG		
Fgfr2 for	CCACAAATGGGCGACTTCCAG	450	NM_010207
Fgfr2 rev	GCAGACAGGGTTCATAAGGC		
Fgfr3 for	GCCGTTTGAGCAGTACTCGC	566	NM_008010
Fgfr3 rev	GAGATGTCGAGCTATGACAC		
Fgfr4 for	GCAGTCTCGGAAGTGCATCC	432	NM_008011
Fgfr4 rev	AGCAGGACCTTGTCCAGAGC		
Cxcl12 for	ACCAGTCAGCCTGAGCTACC	928	L12029
Cxcl12a rev	GGAAGTCAGATGAAGCATGC		
Cxcl12b rev	TGACTTCGAGAGCATTGTGC	873	L12030
Cxcr4 for	TGACTTCGAGAGCATTGTGC	518	NM_009911
Cxcr4 rev	TCCTCTACAGTTCTACAGTC		
Mmp9 for	ATGTGTCCCACTATACCTCC	464	X72794
Mmp9 rev	GACCCAACTTATCCAGACTC		
Fgfr2 for *	CCACAAATGGGCGACTTCCAG	449	NM_010207
Fgfr2 rev *	GCAGACAGGGTTCATAAGGC		
Hprt for *	TCAGGAGAGAAAGATGTGATTGA	124	NM_013556
Hprt rev *	CAGCCAACACTGCTGAAACA		

(*) primers used for real-time PCR

Chapter 3

Interleukin-1 mediated hematopoietic stem cell regulation in the aorta-gonad-mesonephros region of the mouse embryo

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Abstract

Hematopoiesis during development is a highly dynamic process and many factors are thought to be involved in the emergence and regulation of hematopoietic stem and progenitor cells. While previous studies have focused on developmental signalling factors and transcription factors in embryonic hematopoiesis, the role of well-known adult hematopoietic cytokines in the early embryonic generation of the hematopoietic system has been largely unexplored. The cytokine Interleukin-1 (IL-1), best known for its pro-inflammatory properties, has been shown to have radio-protective effects on adult hematopoietic stem cells (HSCs), induce HSC mobilization and increase HSC proliferation and/or differentiation. Here we examine IL-1 and its possible role in regulating hematopoiesis in the mid-gestation mouse embryo. We show that IL-1, IL-1 receptors and signalling mediators are expressed in the mid-gestation aorta-gonad-mesonephros (AGM) region simultaneous to the emergence of HSCs in this site. Moreover, IL-1 signalling is functional in the AGM region, affecting both the hematopoietic supportive microenvironment and hematopoietic cells. *In vivo* analyses show that IL-1 increases AGM hematopoietic progenitor and HSC activity, suggesting that IL-1, via the AGM microenvironment, is an important regulator of hematopoietic progenitors and stem cells in the mid-gestation mouse embryo.

Introduction

The cytokine Interleukin-1 (IL-1) plays a role in a wide range of normal physiological processes and is best known for its role as a major inflammatory mediator. Depending on the target tissue, the cellular context and the local concentration, IL-1 can exert several different biological effects. Within the adult blood system, IL-1 has been implicated as a regulator of hematopoietic stem cells (HSC) and progenitor cells [2,7,12,19]. IL-1 and its high affinity receptor (IL-1 receptor type I and accessory chain) are expressed by bone marrow cells and by populations enriched for HSCs and progenitor cells [24,29,45].

Functional studies show several IL-1 mediated effects on HSCs and progenitor cells, including radioprotection, changes in cell adhesion and migration, and modified cell growth and/or differentiation. The radio-protective effects of IL-1 are demonstrated in the enhanced survival rates of mice injected with IL-1 prior to high dose irradiation. Moreover, HSC activity is maintained when BM cells are treated with IL-1 prior to irradiation [33,37,48]. The increased resistance to cytotoxicity is attributed to IL-1 induced cell cycle effects and IL-1 induced upregulation of the anti-oxidant enzyme Manganese Superoxidase Dismutase (*Sod2*) [4,9]. IL-1 also promotes the mobilization of HSCs to the peripheral blood and affects BM endothelium to enhance the trans-endothelial migration of hematopoietic cells [13,44]. Interestingly, IL-1 regulates the expression of factors shown to play a pivotal role in hematopoietic migration, such as SDF1, CXCR4 and MMP9 [10,11,18,43]. Accelerated hematopoietic recovery upon irradiation is thought to be due to IL-1 induced upregulated production of IL-6, GM-CSF and stem cell factor (SCF or kit ligand) [7,34,37] since IL-1, in combination with hematopoietic growth factors, induces the expansion of hematopoietic progenitor and myeloid precursor cells [2,12,19]. However, some studies report that IL-1 induces differentiation rather than expansion of hematopoietic progenitor cells or that IL-1 completely abrogates HSC activity [25,47]. As these studies were performed with different populations of bone marrow cells, different amounts and combinations of growth factors, it appears that IL-1 has a broad range of biological activities within the hematopoietic system. Despite these many investigations on the role of IL-1 as a regulator of HSCs in the adult, very little is known concerning a role for IL-1 in embryonic hematopoiesis.

During mouse development, the first primitive hematopoietic cells can be found in the yolk sac from embryonic day 7.5 (E7.5) [40]. Shortly thereafter, multipotent hematopoietic progenitors, with the ability to repopulate the hematopoietic system of newborn and hematopoietic deficient recipients but not irradiated adult recipients, are generated in the intra-embryonic para-aortic splanchnopleura [3,46]. At E10.5, the first definitive HSCs with the ability to repopulate adult recipients are autonomously generated in the aorta-gonad-mesonephros (AGM) region and are localized in the endothelium/cell clusters of the ventral aspect of the dorsal aorta (also the vitelline and umbilical arteries) [5,30]. From E11-E12 onwards, definitive HSCs are also detected in the yolk sac, placenta, circulation and fetal liver [14,27,32,39]. The liver is the main fetal hematopoietic organ until birth, when the BM assumes its role as the HSC niche.

The factors that play a role in the regulation of HSCs during development have been a focus of intense research interest. In our search for regulators of AGM HSCs we reported that the gene

Map3k7ip2 (mTAB2) is upregulated between E10 and E11 in the mouse aorta in/near the endothelium [38]. Human TAB2 was originally identified as a binding partner of the MAPK family member TAK1 (TGFβ activated kinase 1) and shown to be involved in IL-1 and TNF signalling [23,42]. TAB2 has been proposed to function as an adapter protein, binding TAK1 to TRAF6 and bringing them to the IL-1 receptor complex, resulting in NFκB and JNK activation [23,42]. Since the expression pattern of mTAB2 in the dorsal aorta endothelium correlates with the emergence of HSC activity in this region, we set out to investigate whether IL-1 is a regulator of HSCs in the mid-gestation embryo.

Here we show that all the essential molecules of the IL-1 signalling pathway are expressed and that IL-1 signalling is functional in the AGM region from E11 onwards. IL-1RI expression is localized to the ventral aspect of the dorsal aorta and is expressed by hematopoietic, endothelial and mesenchymal cells, as well as by AGM stromal cell lines. We show further that IL-1 induces an increase in AGM hematopoietic cells and numbers of myeloid progenitors and HSCs. Thus, IL-1, generally thought to be an adult cytokine, plays a role in the regulation of HSCs in the mid-gestation mouse embryo.

Material and Methods

Embryo generation and cell culture

Animals were housed according to institutional guidelines, with free access to water and food. Animal procedures were carried out in compliance with the Standards for Humane Care and Use of Laboratory Animals. To generate embryos, matings were set up between (C57/BL10xCBA)F_1 females and Ln72 human β-globin [41] males and between C57/BL6 females and Act-GFP males [35] and between *Il1r1*$^{-/-}$ (*Il1r* $^{tm1Imx/\ tm1Imx}$ [17]) males and females. *Il1r1*$^{-/-}$ (*Il1r* $^{tm1Imx/\ tm1Imx}$) mice were obtained form Jackson Labs and genotyped according to provided directions. The day of the vaginal plug was counted as day 0. Pregnant mice were sacrificed by cervical dislocation and AGM regions and livers were dissected as described [6]. For obtaining single cell suspensions of embryonic material, tissues were collagenase treated (0.125% in PBS/10% FCS) for 1 hour at 37°C.

3T3 fibroblasts were cultured in DMEM/ 10% FCS/ 1% PenStrep and for some experiments overnight serum starved with DMEM/ 1% FCS. Cell suspensions from several AGM (or liver) tissues were pooled and equivalents of 2-4 tissues were seeded in 6 well plates in DMEM/10%FCS/ 1% PenStrep. The next day cells were stimulated with IL-1β for target gene induction and IκB degradation studies.

UG26-1B6 cells were cultured at 33°C in LTC-SM medium containing 50% M5300 (Stem Cell Technologies)/15% FCS/35% alpha-MEM/1% PenStrep/10μM β-mercaptoethanol and stimulated with 10 ng/ml IL-1β for 2, 6 and 24 hours. Cells were lyzed in TRIZOL and RNA was isolated and cDNA generated as described.

Immunohistochemistry

Embryos were snap-frozen in TissueTek (Sakura) and 7-10μM cryosections generated. Sections were fixed in 2% paraformaldehyde/PBS and endogenous peroxidase activity was blocked with 1.2%

H_2O_2/ MeOH. Sections were blocked with TSA blocking buffer and incubated with IL-RI antibody (clone 12A6; BD biosciences) overnight at 4°C. Subsequently, sections were incubated with α-rat-biotin antibody and streptavidin-HRP (DAKO) in combination with TSA biotin system (PerkinElmer) according to provided instructions. Staining was visualized with DAB chromogen (Sigma) and sections counterstained with hematoxylin. Sections were embedded with Entallan (Merck). Pictures were taken with an Olympus BX40 microscope.

Explant cultures and *in vivo* transplantation assays for HSC activity.

E11 AGM and liver tissues (marked with GFP or human β-globin) were dissected and 3 day explant cultures were performed as described previously [30] in the presence of 0, 1 or 10 ng/ml IL-1β (PeproTech Inc.). After the culture single cell suspensions from pooled AGMs were obtained and different cell dilutions (measured as embryo equivalents) were injected intravenously together with non-marked spleen cells ($2x10^5$) into 9.5 Gy irradiated (C57BL/10xCBA)F_1 recipient mice. AGM cells from *Il1r1*$^{-/-}$ and Act-GFP transgenic embryos were transplanted into (129SVxC57BL/6) adult recipients. Repopulation was assayed at 1 and 4 months post-transplantation by donor specific semi-quantitative PCR (human β-globin or GFP) on peripheral blood DNA as described previously [5,30,41]. Only mice with >10% engraftment were considered repopulated. For multi-lineage repopulation analysis DNA was isolated from spleen, thymus, bone marrow and peripheral blood or from FACS-sorted cells from these tissues and assayed for donor contribution by PCR.

In vitro progenitor colony assay

After 3 day AGM explant culture in the presence or absence of IL-1β, single cell suspension from a pool of 3-4 AGM tissues were made. Cells were seeded at a concentration of $0.5x10^4$ to $5x10^4$ cells per plate in methylcellulose medium (Methocult GF M3434; Stem Cell Technologies Inc.) supplemented with stem cell factor (SCF), IL-3, IL-6 and Epo. All cultures were incubated at 37°C in a humidified chamber under 5% CO_2. Colony-forming unit-granulocyte (CFU-G), colony-forming unit-macrophage (CFU-M), colony-forming unit-granulocyte macrophage (CFU-GM) and colony-forming unit-granulocyte erythroid megakaryocyte macrophage (CFU-GEMM) were scored with an inverted microscope at day 7 of the culture.

FACS analysis

Single cell suspensions were stained with IL-1RI-phycoerythrin (clone 35F5) in PBS/10% FCS/ 1%PenStrep on ice for 20-30 minutes. Cells were co-stained with FITC-labelled antibodies for c-kit (CD117), Mac1 (CD11b), CD45 or CD31. Other FACS analyses were performed with CXCR4, followed by Streptavidin-PE (Caltag) or anti-rat-PE and AnnexinV-FITC antibodies. Dead cells were excluded by 7AAD (Molecular Probes) and FACS analysis was performed on FACScan (Becton Dickinson) and analysed with Cell Quest software. All antibodies used for FACS analysis are from BD biosciences.

RNA isolation, cDNA synthesis and RT-PCR analysis

Total RNA was isolated with TRIZOL (Gibco/ Life Technologies) according to manufacturer's instructions and DNAse treated with RQ1 RNase free DNAse (Promega). For cDNA synthesis 1-5 μg of total RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen/Life Technologies) according to manufacturer's instructions. PCR reactions were performed in 50 μl with 1U Amplitaq (PerkinElmer) and provided buffer, 100 ng of each primer, 1mM dNTPs, and 2 μl of cDNA. PCR cycles as follows: 5 minutes 92°C, 27-40x (40 sec 92°C, 40 sec 58°C, 1-2 minutes 72°C), 7 minutes 72°C. PCR primers are listed in Supplementary Table 1. For semi-quantitative RT-PCR analysis several cDNA dilutions were used for RT-PCR. PCR products were run on 1.2% agarose/1xTBE gels with ethidium bromide and scanned on a Typhoon scanner (Molecular Dynamics) and analysed with ImageQuant software.

IL-1 induced target gene induction and IκB degradation

3T3 cells were treated with 10 pg/ml-100 ng/ml IL-1β (PeproTech Inc.) for indicated times. Cells were scraped from tissue culture plates in cold PBS, collected by centrifugation and lysed in TRIZOL. cDNA synthesis and RT-PCR analysis as described. Cells from AGM (or liver) tissues were stimulated with IL-1β for indicated times and all cells (non-adherent and adherent) were harvested for RNA isolation and RT-PCR analysis.

To examine if gene induction was independent of protein synthesis, cells were pre-treated with cyclohexamide (Sigma) at a final concentration of 10μg/ml for 1 hour prior to IL-1β stimulation. For blocking IL-1β, 2000 fold excess (200ng) IL-1 blocking antibody (R&D systems) was incubated with IL-1β (100 pg) for 2 hours on ice prior to addition to the cells.

For IκB degradation studies, E11-E12 AGM or liver cells or 3T3 cells were stimulated with 10 ng/ml and 100ng/ml IL-1β respectively for 0-30 minutes. Protein lysates were made with RIPA lysisbuffer, separated on 10% SDS-PAGE and blotted against PVDF membrane (Millipore). Blotted membranes were blocked with 4% non-fat milk (Biorad)/TBS-T and incubated with IκB antibody (Cell signalling Technology) followed by α-Rabbit-HRP (DAKO) and visualized with ECL detection.

Results

IL-1 signalling components are expressed and function in the mid-gestation AGM

To investigate whether IL-1 plays a role in regulating hematopoiesis in the AGM region, cells from this mid-gestation tissue were examined for expression of IL-1 receptors, IL-1 signalling components and the ligand IL-1. RT-PCR analysis (Figure 1A) shows the transcription of IL-1 receptors, *Il1r1* (receptor type I) and *Il1rap* (Receptor Associating Protein) in the E11 and E12 AGM and fetal liver (FL). *Il1rap* expression is already initiated at E10. The non-signalling *Il1r2* (receptor type II) is detected from E11 onwards in the AGM and FL. Intracellular signalling components, *Map3k7ip2* (TAB2) and *Map3k7* (TAK1) are also expressed in the AGM and FL at E11 and onwards. Other essential IL-1R

signalling components, *Traf6* and *Irak4*, are expressed earlier, from E10 onwards, in both AGM and FL. Moreover, *Il1β* is expressed in the AGM region beginning at E11 and increased at E12. Thus, all the essential IL-1 signalling components are expressed in the E11-E12 AGM and FL.

The functional responsiveness of AGM and FL cells to IL-1 was measured by target gene induction (*Junb* and *Sod2*) and NFκB pathway activation (IκB degradation) after IL-1 stimulation. The conditions for the transcriptional induction of *Junb* by IL-1β were first determined in embryonic 3T3 fibroblasts. As determined by RT-PCR, 10 ng/ml IL-1β yielded a 2.9 fold increase in *Junb* expression after 2 hours of IL-1β stimulation (Supplementary Figure 1). When stimulated in the presence of an IL-1β-neutralizing antibody, *Junb* levels were reduced to those observed in untreated 3T3 cells (Supplementary Figure 1), thus demonstrating the specificity of the assay. We then prepared cell suspensions of dissected E10, E11 and E12 AGM and FL tissues and cultured the cells overnight prior to IL-1β stimulation (Figure 1B). When treated for 30 minutes to 2 hours with IL-1β, *Junb* expression rapidly increased in E11/E12 AGM and FL cells (range 1.7-60 fold) as compared to untreated cells (Figure 1C). Consistent with the observation that the IL-1 receptor is not expressed on E10 AGM cells, we did not detect IL-1β induced *Junb* expression in E10 AGM cells (data not shown). IL-1β also induced the upregulation of *Sod2* gene in AGM cells (2.5-8 fold). Thus, IL-1 target genes *Junb* and *Sod2* are transcriptionally upregulated in E11/E12 AGM cells treated with IL-1β.

IL-1β mediated effects on the NFκB pathway were also tested in this culture system by Western blot analysis for IκB. We observed IκB degradation in both E12 liver and 3T3 cells (Figure 1D) and a moderate reduction of IκB protein in E11/E12 AGM and E11 FL (data not shown) following IL-1β stimulation. Thus, a population of cells within the E11/ E12 AGM and FL are IL-1 responsive and display functional IL-1 signalling properties.

Interleukin-1 receptor expression is localized to the mid-gestation dorsal aorta

We next focused on the AGM region to determine in which cell lineage the IL-1RI is expressed. Immunohistochemistry, flow cytometric analysis and RT-PCR were performed. Immunostaining on E11 transverse sections localize IL-1RI expression to the cells (endothelial/hematopoietic) along the ventral wall of the aorta and cells (mesenchymal) directly underlying the lumen (Figure 2A). At E11, expression is low and limited to the cells on the ventral aspect of the aorta, while at E12 IL-1RI expression is found in the same cells but expression is spread around the entire aorta (not shown). The low IL-1RI staining precluded clear determination of expression in the hematopoietic lineage, particularly in hematopoietic clusters closely associated with the aortic endothelium.

Flow cytometric analyses revealed that on average approximately 0.16% and 0.33% of E11 and E12 aorta cells express IL-1RI (Table 1). Multicolour flow cytometry showed that on average about 45% of the E11 IL-1RI positive cells express CD45 or c-kit (Figure 2B). Thus, while the localized expression of IL-1RI by immunohistology suggests that many of these cells are of the endothelial and mesenchymal lineages, flow cytometry indicates that some of these cells are also of the hematopoietic lineage, or possibly endothelial cells taking on hematopoietic fate. To confirm the expression of IL-1RI in the different lineages, E11 aorta cells were sorted based on hematopoietic (c-kit$^+$CD34$^+$), endothelial (VE-cadherin$^+$CD45$^-$) or

mesenchymal (VE-cadherin$^-$CD45$^-$) marker phenotypes and examined by RT-PCR for the expression of *IL1r1*, *IL1r2* and *IL1rap.* As shown in Figure 2C, cells of all three lineages express the *IL1r1* and *IL1r2* genes but to varying degrees. HSCs appear to express less *IL1r1* and more *IL1r2*, than endothelial or mesenchymal cells. HSCs and endothelial cells also express *IL1rap* (mesenchymal cells not tested). The lack of a positive selection method for mesenchymal cells from the AGM prompted us to examine several well-characterized E11 AGM derived mesenchymal cell lines for expression of *IL1r1* and *IL1rap*. RT-PCR revealed *IL1r1* and *IL1rap* expression in all tested embryonic stromal cell lines (Table 2). Flow cytometric analysis shows a high expression of IL-1RI on one of the best hematopoietic supportive AGM stromal lines, UG26-1B6 (Figure 2D). Thus, IL-1RI is expressed on some hematopoietic, endothelial and mesenchymal cells in the mid-gestation aortic region.

IL-1 increases the number of hematopoietic cells in the E11 AGM

To test the functional effects of IL-1 signalling on AGM hematopoiesis, we performed flow cytometric analysis for hematopoietic markers on cells from E11 AGM explants cultured in the presence or absence of IL-1β. We observed IL-1β-induced increases in the percentages of CD45$^+$ and c-kit$^+$ cells in the cultured AGM tissues (Figure 3A). The percentage of CD45$^+$ cells increased on average 1.4- and 1.9-fold and c-kit$^+$ cells increased 1.3- and 1.8-fold when cultured in the presence of 1 ng/ml and 10 ng/ml IL-1β respectively. Similar increases in the percentage of c-kit$^+$ cells were found in E11 aorta subregions cultured in the presence of IL-1β.

To test whether IL-1β affects AGM hematopoietic progenitor cells, methylcellulose cultures were performed after AGM organ culture in the presence or absence of IL-1β. In the presence of 1 ng/ml IL-1β the total number of colony-forming units (CFU) per AGM was increased 1.3-fold as compared to the control cultures (not shown). Since IL-1 in the adult is known to promote myelopoiesis, we focused our analysis on CFU-G and CFU-M, the most committed myeloid progenitors and CFU-GEMM, the most immature myeloid progenitor. As shown in Figure 3B, the number of CFU-G per AGM was increased in a dose-dependent manner in the presence of IL-1β. Also, the number of CFU-M per AGM was increased with both doses of IL-1β. Consistent with this increase in myeloid restricted progenitors, we observed an increase in the percentage of Mac1$^+$ (CD11b) cells in AGM explants cultured in the presence of IL-1 (Figure 3C). Interestingly, while there is a small increase in CFU-GEMM in AGM explants treated with 1 ng/ml IL-1β (2.5 to 4- fold), these immature progenitors are decreased in AGM explants treated with high dose IL-1β. Thus, low dose IL-1β increases the number of immature and mature myeloid hematopoietic progenitors in AGM explants, while high doses regulate myelopoiesis in a positive manner but act to decrease the number of immature hematopoietic progenitors.

IL-1 affects hematopoietic stem activity in the E11 AGM

To test whether IL-1β affects AGM HSCs we performed *in vivo* transplantation assays with cells from E11 AGM explants cultured in the absence or presence of IL-1β. At 4 months post-injection, the peripheral blood DNA of transplantation recipients was analysed for the donor genetic marker by semi-quantitative PCR. Only recipients with high-level donor chimerism (greater than 10%) were con-

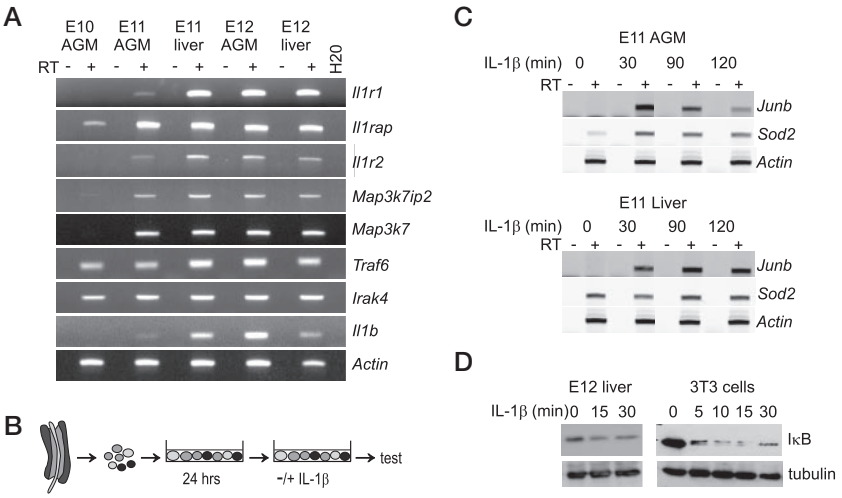

Figure 1. IL-1R signalling components are expressed and functional in the mid-gestation AGM region and liver

(A) RT-PCR analysis performed to examine the expression of the IL-1 receptors type I (*Il1r1)*, the accessory receptor (*Il1rap*) and the receptor type II (*Il1r2*) and several downstream signalling components, including *Map3k7ip2* (TAB2), *Map3k7* (TAK1), *Traf6* and *Irak4* and the ligand IL-1β (*Il1b*) in the E10-E12 AGM region and E11-E12 fetal liver. **(B)** Overview of the culture method used to study gene induction or IκB degradation in AGM tissues. Single cell suspensions were made from E11 AGM tissues and cultured in 6 well plates overnight. The next day, cells were treated with IL-1β and harvested for RT-PCR analysis or IκB degradation studies. **(C)** Representative semi-quantative RT-PCR for the IL-1β target genes *Junb* and *Sod2* (MnSOD) after stimulation of E11 AGM and liver single cell suspensions with IL-1β (10 ng/ml) for 0, 30, 90 or 120 min. RT=reverse transcriptase. **(D)** Western blot showing rapid IκB degradation after IL-1β stimulation of E12 liver cells (left panel) or 3T3 fibroblasts (right panel).

sidered repopulated. As shown in Figure 4A, AGM explants cultured with a low concentration of IL-1β (1 ng/ml) showed a small increase in HSC activity (69% of recipients repopulated) as compared to control AGM cultures (54% of recipients repopulated). High dose IL-1β (10 ng/ml) severely decreased HSC activity, consistent with the decrease in CFU-GEMM. Hence, low levels of IL-1β have a positive effect on HSC activity, while high levels of IL-1β severely disrupt AGM HSC activity.

The physiologic relevance of IL-1 signalling in the AGM region was next examined in embryos deficient for IL-1RI. Cells from E11 AGM regions of *Il1r1*$^{+/+}$ and *Il1r1* $^{-/-}$ embryos were either directly transplanted into irradiated adult recipients to test for HSCs or transplanted after 3-day explant culture. Previous studies have shown that AGM HSCs are amplified during the culture period, and hence such explants should reveal whether IL-1RI signalling affects HSC expansion. Both the direct transplantation and transplantation after explant culture reveal a decrease in AGM HSCs in *Il1r1* $^{-/-}$ embryos as compared to *Il1r1*$^{+/+}$ (Figure 4B). Calculation of the number of HSCs in the directly transplanted AGMs revealed 1.12 HSC/ *Il1r1*$^{+/+}$ AGM and 0.45 HSC/ *Il1r1*$^{-/-}$ AGM (more than 2-fold decrease in HSC number). There appears to be an even greater decrease in HSCs in the *Il1r1* $^{-/-}$ AGMs after explant culture as compared to *Il1r1*$^{+/+}$ explants, strongly suggesting defective HSC amplification.

Table 1. Percentages of IL-1RI, CD45 and c-kit expressing aorta cells

	E11 Aorta		E12 Aorta	
	mean	(range)	mean	(range)
IL-1RI$^+$/CD45$^-$	0.09	0.05-0.11	0.22	0.12-0.43
IL-1RI$^+$/CD45$^+$	0.07	0.05-0.11	0.11	0.01-0.16
IL-1RI$^-$/CD45$^+$	3.99	3.83-4.08	3.25	2.81-3.98
IL-1RI$^+$/c-kit$^-$	0.09	0.07-0.12	0.16	0.14-0.19
IL-1RI$^+$/c-kit$^+$	0.06	0.03-0.09	0.15	0.03-0.22
IL-1RI$^-$/c-kit$^+$	6.60	4.86-9.83	8.35	5.31-13.05

For E11, n=3 and for E12, n=4

Table 2. Expression of IL-1 signalling molecules by embryonic stromal cell lines

Cell line	Origin	Expression					
		IL-1RI *(Il1r1)*	IL-1RacP *(Il1rap)*	IL-1RII *(Il1r2)*	IL-1α *(Il1a)*	IL-1β *(Il1b)*	Actin *(Actb)*
AM20-1A4	Ao	++	++	++	-	-	++
AM30-2A4	Ao	++	++	±	-	-	++
AM30-3F4	Ao	++	++	-	-	-	++
AM30-3A3	Ao	++	++	+	-	-	+
UG26-1B6	UGR	++	++	-	-	-	+
EL08-1D2	FL	++	++	-	-	-	+
EL23-1C2	FL	++	++	-	-	-	+
FBMD-1	BM	++	++	-	+	-	++

IL-1 receptor (IL-1RI and IL-1RAcP) expression is detected by RT-PCR in several stromal cell lines derived from different embryonic and adult mouse tissues. No IL-1β expression could be detected in these cell lines and IL-1α expression was only detected in the adult BM-derived FBMD-1 stromal cell line. Expression of the IL-1 decoy receptor (IL-1RII) was detected in some of the stromal cell lines tested. BM: bone marrow; Ao: dorsal aorta from AGM region; UGR: urogenital ridge from AGM region; FL: fetal liver. Expression levels of these transcripts are indicated by their relative expression levels, with ++ indicating very high expression; + high expression; ± low expression and – no expression was detected.

HSC multilineage engraftment was tested in recipients of *Il1r1* $^{-/-}$ E11 AGM cells. High donor cell chimerism (32-100%) was found in all hematopoietic tissues and in all hematopoietic lineages tested (Figure 4C). Moreover, high-level repopulation of secondary recipients revealed that the *Il1r1* $^{-/-}$ E11 AGM HSCs self-renew (data not shown). Thus, lack of signalling through IL-1RI affects the number but not potential of AGM HSCs, and suggests a role for IL-1 in the normal physiologic growth and amplification of these cells in the embryo.

IL-1 affects regulators of hematopoietic cell migration

We next tested whether the IL-1β mediated affects on the hematopoietic function of AGM explants corresponded to changes in the levels of expression (autoregulation) of IL-1 receptors, ligands and/or changes in the expression of other downstream effectors (those affecting the mobilization of HSCs).

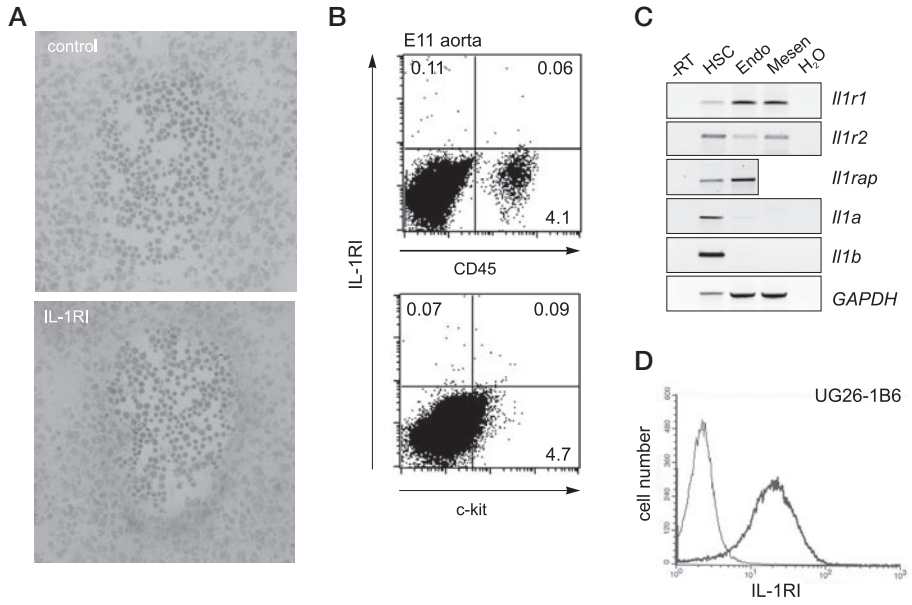

Figure 2. IL-1RI is expressed by E11 aortic haematopoietic, endothelial and mesenchymal cells
(A) Immunostaining performed with an IL-1RI specific (bottom panel) or control (top panel) antibody on transverse cryosections showing the dorsal aorta of the AGM region from E11 embryos. Dorsal side of tissue is at the top and ventral side is at the bottom of each section. Original magnification is 200x. (colour print, see back side cover) **(B)** Representative flow cytometric dot plots showing E11 aorta cells (n=4) stained with antibodies specific for IL-1RI and CD45 (top panel) or c-kit (bottom panel). Percentages of cells in each quadrant are indicated. 3-3.6 x 10^4 events are shown and over 5 x 10^4 events were analysed. **(C)** RT-PCR analysis for IL-1 receptor and ligand expression in sorted HSCs ($CD34^+c\text{-}kit^+$), endothelial cells ($CD45^-VE\text{-}cadherin^+$; Endo) and mesenchymal cells ($CD45^-VE\text{-}cadherin^-$; Mesen) from the E11 AGM. *GAPDH* expression serves as the normalization control. –RT = no reverse transcriptase control. **(D)** Flow cytometric analysis of IL-1RI specific expression on UG26-1B6 stromal cells. x-axis indicates intensity of fluorescent signal from staining with IL-1RI specific antibody and y-axis indicates the number of cells.

Since it has been proposed that IL-18 and IL-1 signalling may overlap, we also examined changes in IL-18 receptor and ligand expression. We performed semi-quantitative RT-PCR for some of these genes on E11 AGMs before culture and following explant culture in 0, 1 or 10 ng/ml IL-1β. As shown in Figure 5A, *IL1a*, *IL18r1, IL18rap* and *IL18* expression are increased as a result of AGM explant culture. Culture of AGM explants in the presence of IL-1β does not further affect the expression levels of *IL1r1*, *IL1rap* or *IL1a*, but does increase *IL1r2* and *IL1b* expression particularly at the 10 ng/ml dose. Interestingly, in the presence of 1 ng/ml IL-1β higher levels of *IL18r1* and decreased *IL18rap* and *IL18* expression are found, while at 10 ng/ml *1l18rap* is greatly increased. Thus, the expression changes for some of these regulatory molecules are culture and IL-1β dose dependent and may mediate hematopoietic function.

Since IL-1 is known to affect the mobilization of adult BM HSCs and other adult hematopoietic cells, we examined whether IL-1 could affect the expression of mobilization regulators in AGM cells and the AGM stromal line UG26-1B6. We performed semi-quantitative RT-PCR for *Cxcl12* (SDF1),

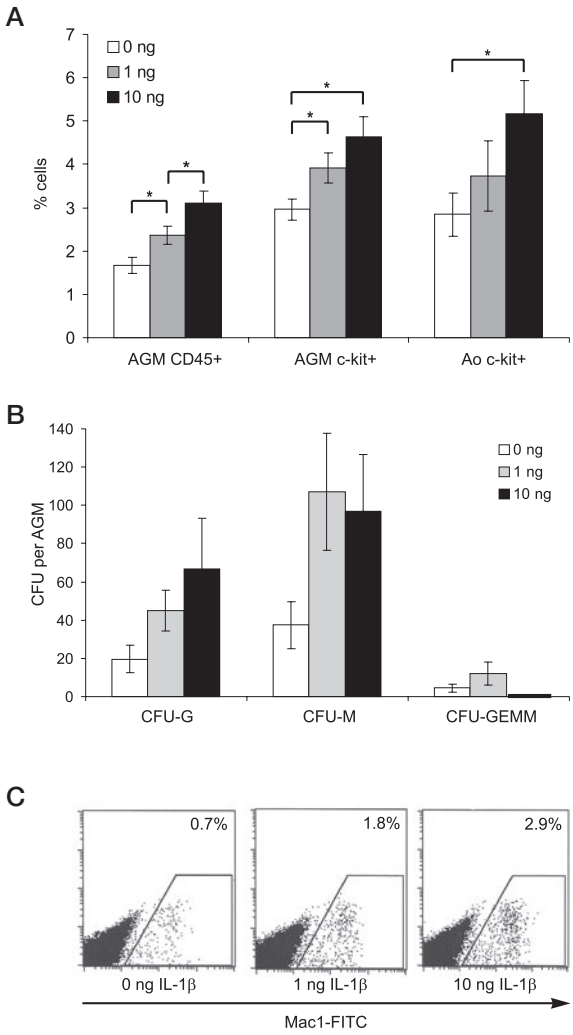

Figure 3. IL-1β increases hematopoietic cells in the E11 AGM region

E11 AGM tissues were cultured for 3 days in the presence or absence of different doses of IL-1β. **(A)** Flow cytometric analysis showing percentage of cells positive for CD45 or c-kit after AGM explant culture (n=5) and Aorta (Ao) explant culture (n=4). The error bars indicate the SEM. Asterisks * indicate statistical significance ($p \leq 0.05$) by t-test. Average (± SEM) cell counts (x 10^5) for aorta explants in 0, 1 and 10 ng/ml IL-1β are 2.5 ± 0.2, 2.3 ± 0.3 and 2.0 ± 0.8 respectively. The absolute number of AGM hematopoietic cells increased 1.4 to 1.7 fold in the presence of IL-1β. **(B)** Number of CFU-G, CFU-M and CFU-GEMM per E11 AGM explant cultured for 3 days in the presence of 0, 1, or 10 ng/ml IL-1β. Colonies were scored after 7 days of methylcellulose culture (n=2). IL-1β increases the number of CFU-G and CFU-M per AGM at both 1 and 10 ng/ml. The number of CFU-GEMM colonies per AGM was highest at a dose of 1 ng/ml IL-1β and was zero at 10 ng/ml. **(C)** Representative flow cytometric dot plot showing an increased percentage of $Mac1^+$ cells in AGM organ cultures in the presence of IL-1β. Percentage of cells in gated area is indicated. On average a 1.7 fold and 2.4 fold increase in the percentage of $Mac1^+$ cells was observed in the presence of 1 ng/ml and 10ng/ml IL-1β respectively (n=5). 2.1-2.7 x 10^4 events are shown.

Cxcr4 and *Mmp9* on cDNA from E11 AGM tissues before and after explant culture in the presence or absence of IL-1β. As shown in Figure 5B, IL-1β downregulates *Cxcl12a* and *b* expression (SDF1α and β isoforms) in explants in an IL-β dose dependent manner. *Cxcr4* expression appears to be unchanged or slightly increased in the AGM explants. *Mmp9* is induced in AGM explants, with the highest level of induction in explants cultured with 1 ng/ml IL-1β. To more specifically examine the expression of CXCR4 on single E11 AGM cells, we performed FACS analysis on aorta explants cultured in the presence or absence of IL-1β. A dose dependent increase in the percentage of $CXCR4^+$ cells was found (Figure 5C), representing a 1.3- and 2-fold increase in the presence of 1 ng/ml and 10 ng/ml IL-1β respectively. Thus, in the E11 AGM and more specifically the aorta subregion, IL-1β affects the expression of regulators known to play an important role in HSC migration.

We next stimulated UG26-1B6 stromal cells for 2 to 24 hours with IL-1β and examined the temporal expression of several hematopoiesis-related genes by RT-PCR. IL-1β stimulation of UG26-1B6 cells did not change the expression level of the *Il1r1* or the *Il1rap*, but induced *I11a* expression in a transient manner. No expression of *Il1b* was detected prior to or after IL-1β stimulation (not shown). As shown in Figure 5D, no changes were detected in *Il6* expression. Prolonged IL-1β stimulation resulted in downregulation of *Kitl* (SCF), *Cxcl12b* (SDF1β*)* and *Csf2* (GM-CSF; not shown). Interestingly, *Cxcl12a* (SDF1α*)* was upregulated early in the culture period and then downregulated by 24 hours. *Csf1* (M-CSF) expression was slightly upregulated during the short period of IL-1β stimulation (not shown). Together with the results of AGM and aorta explants, the IL-1β induced changes in the AGM stromal cell line suggest a complex regulation of molecules and interacting cell types in the AGM to control hematopoietic cell growth and migration.

Discussion

We have shown here that a well-known adult cytokine, IL-1 and the molecules essential in its signalling pathway are expressed and active in the early development of the hematopoietic system. Their expression in the aortic subregion of the AGM suggests that IL-1 signalling plays some role in hematopoietic regulation. We have shown through functional analyses that IL-1 increases AGM HSCs and hematopoietic progenitors while HSCs are decreased in *IL1r1*$^{-/-}$ AGMs. Such a role for IL-1 in the embryo was unexpected, since gene-targeting experiments show no obvious phenotype in adult mice. IL-1 (α and β) and IL-1RI deficient mice are viable and under steady state conditions do not display an obvious hematopoietic phenotype [16,21]. However, when the immune system of these deficient mice is challenged, inflammatory responses and cytokine expression are reduced. Our demonstration of a role for IL-1 in the developing AGM HSC compartment lends credence to the notion that tissue development in the embryo and repair due to trauma in the adult are regulated similarly. Also, while it is thought that in the adult there is functional redundancy between IL-1 and IL-18 [8,15], the fact that no *Il18* and only very low levels of *Il18r1* are expressed in the E11 AGM region most likely allowed us to observe the physiologic role of IL-1 signalling in regulating HSC numbers in the E11 AGM region.

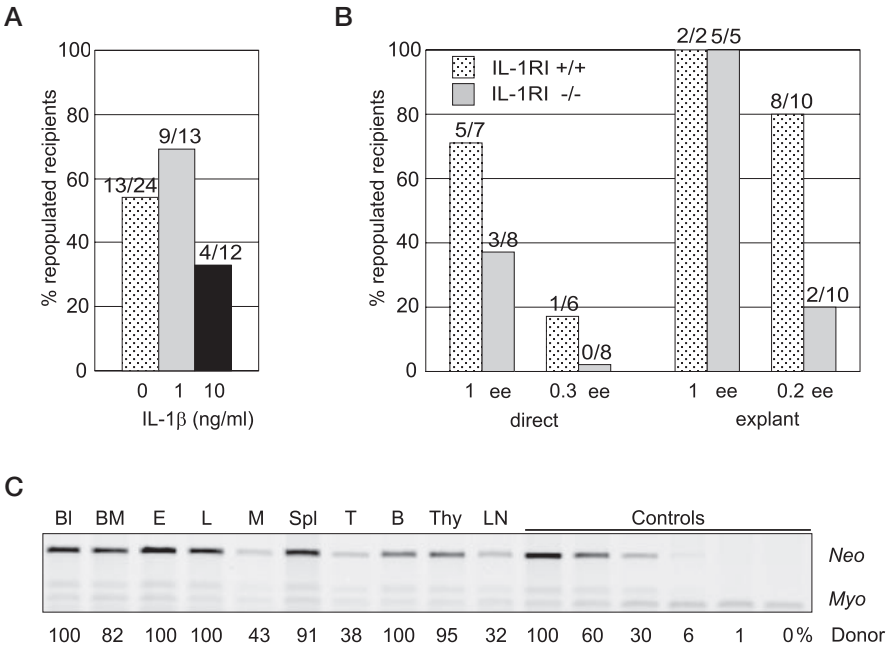

Figure 4. IL-1 receptor signalling affects AGM HSCs

(A) Percentage of adult recipient mice repopulated with donor E11 AGM cells cultured in the presence of IL-1β. E11 AGM explants were cultured in 0, 1 or 10 ng/ml IL-1β for 3 days and cells were injected into irradiated recipients (1 embryo equivalent (ee)). At four months post-transplantation, recipient peripheral blood DNA was analysed for donor hematopoietic chimerism by semi-quantitative PCR. Indicated above each column is the number of mice repopulated per number of recipients transplanted. Only mice with >10% donor chimerism were considered repopulated. Combined results of 8 separate transplantation experiments. **(B)** Percentage of adult recipient mice repopulated with HSCs from *Il1rI*$^{+/+}$ and *Il1rI* $^{-/-}$ AGM regions. E11 AGM cells (1, 0.3 or 0.2 ee) were either transplanted directly (direct) or following 3 days of explant culture (explant) into irradiated adult recipients. At four months post-transplantation, peripheral blood DNA of recipients was analysed for donor hematopoietic chimerism by semi-quantitative PCR. Indicated above each column is the number of mice repopulated per number of recipients transplanted. Only mice with >10% donor chimerism were considered repopulated. Combined results of 4 separate transplantation experiments. **(C)** Representative semi-quantitative PCR analysis for multilineage hematopoietic repopulation of a recipient transplanted with *Il1rI* $^{-/-}$ E11 AGM explant cells four months post-transplantation. Percentage donor marker contributions (*Neo* gene of the *Il1rI* $^{-/-}$ locus) are indicated below each lane. Controls include the *Myo* gene DNA normalization and quantitation standards containing 100, 60, 30, 6, 1 and 0% donor (*Neo*) DNA. BL=blood; BM=bone marrow; E=erythroid; L=lymphoid; M=myeloid; Spl-spleen; T=T lymphocyte; B=B lymphocyte; Thy=thymus; LN=lymph node.

Does IL-1 act on the cells of the AGM hematopoietic microenvironment?

By immunostaining we found that IL-1R expressing cells localize mainly on the ventral side of the dorsal aorta at E11 and by E12 expression extends to a few more cells around the aorta. While some of the IL-1R expressing cells are endothelial and hematopoietic, the majority of aortic cells expressing IL-1R are mesenchymal, as defined by the absence of expression of VE-cadherin, CD45 and CD31. In support of these data, we and Oostendorp et al. have shown that stromal cells derived from the AGM

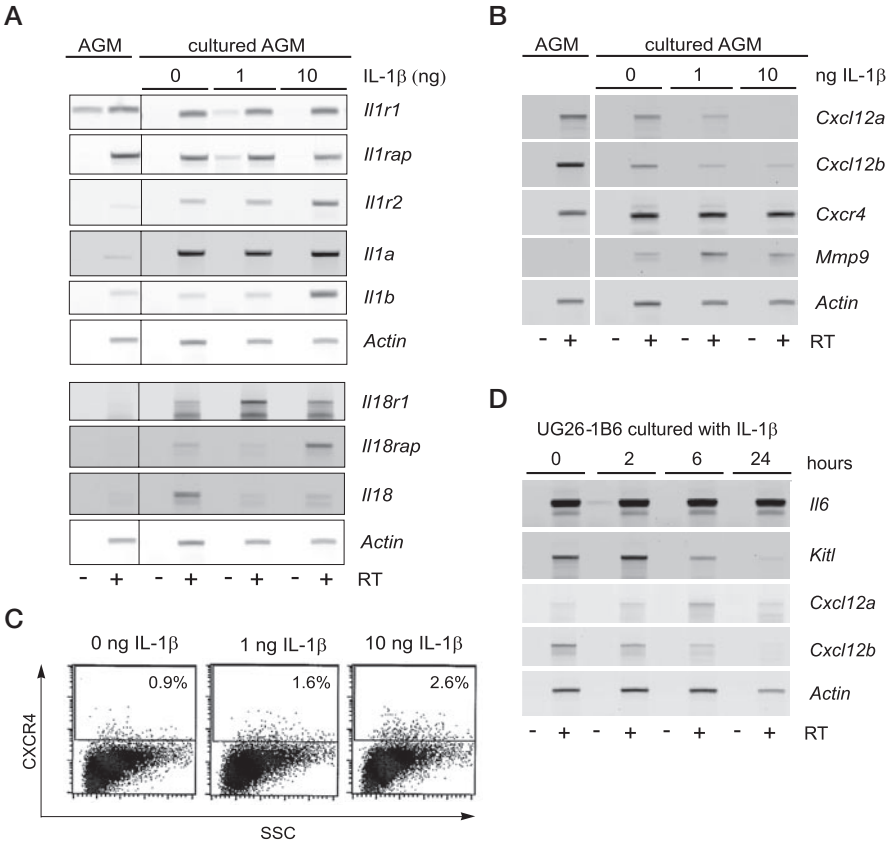

Figure 5. Expression of IL-1 signalling molecules and hematopoietic regulators in the IL-1β stimulate AGMs

(A) RT-PCR analysis of E11 AGM tissue before and after 3 day explant culture in the presence of 0, 1 or 10 ng/ml IL-1β. Changes in gene expression of some of the tested genes (*Il1r1, Il1rap, Il1r2, Il1a, Il1b, Il18r1, Il18rap, Il18)* are found after explant culture or are induced by the presence of IL-1β. **(B)** RT-PCR analysis of E11 AGM tissue before and after 3 day explant culture in the presence of 0, 1 or 10 ng/ml IL-1β. Changes in gene expression of some of the tested genes (*Cxcl12 a* and *b, Cxcr4, Mmp9*) are found after explant culture or are induced by the presence of IL-1β. **(C)** Flow cytometric analysis of cultured E11 aorta explants for expression of CXCR4. E11 aorta explants were cultured in the presence of 0, 1 or 10 ng/ml IL-1β for 3 days prior to analysis. The percentage of $CXCR4^+$ cells is indicated in the gated upper section (n=3). 3 x 10^4 events were analysed and 1.3-1.5 x 10^4 events are shown. **(D)** RT-PCR analysis of UG26-1B6 cells treated with 10 ng/ml IL-1β for various times (2-24 hours) and examined for gene expression of several hematopoietic cytokines (*Il6, Kitl)* and chemokines (*Cxcl12a, Cxcl2b)*. Representative experiments are shown in A, B and D (n=2). *Actin* was used as a cDNA normalization control. –RT = no reverse transcriptase; +RT = +reverse transcriptase.

express the IL-1R [36]. Moreover, Oostendorp et al. have shown that high level IL-1R expression by AGM stromal cells correlates with long-term support of enriched HSCs [36]. These AGM stromal cells are of the mesenchymal lineage. Stimulation of these stromal cells by IL-1 induces the regulation of

molecules involved in the mobilization of HSCs. Interestingly, the AGM stromal cells do not express IL-1, suggesting that stimulation of the IL-1 pathway is cell extrinsic.

Our RT-PCR analysis reveals high-level expression of both IL-1α and IL-1β by HSCs and some low level expression by endothelial cells (Figure 2C). In preliminary studies we have localized rare IL-1 expressing cells in the endothelial and directly underlying cell layer of the E12 aorta. We have also found small aggregates of IL-1 producing cells ventral to the notochord and ventral-lateral to the dorsal aorta (unpublished data). Hence, IL-1 signalling may occur through direct interactions of IL-1 producing aorta cells with the IL-1R$^+$ stromal microenvironment or through secretion of IL-1 through the AGM interstitium. As we have shown that the components of the IL-1 signalling pathway, the type I and accessory receptor and several intracellular mediators are all expressed in the AGM, we suggest that the IL-1 signalling cascade is triggered in stroma (mesenchyme and/or endothelium) to effect changes in the expression and elaboration of molecules regulating the proliferation, differentiation and mobilization of AGM hematopoietic cells (Figure 6). Since the expression of the majority of IL-1 signalling molecules is not seen at E10, IL-1 probably does not play a role in hematopoietic stem/progenitor cell emergence.

IL-1 in the proliferation and/or differentiation of AGM hematopoietic cells

In the adult, IL-1 is known to affect several cellular processes, including proliferation, differentiation and apoptosis (reviewed in [7]). We examined the possible effects of IL-1 on these processes in AGM hematopoietic cells. IL-1β was able to increase the numbers of CD45$^+$ hematopoietic cells and c-kit$^+$ hematopoietic progenitors in a dose-dependent manner. Moreover, we observed in *in vitro* progenitor assays that IL-1 increases the number of CFU-G and CFU-M per AGM organ culture and at a low dose, increase CFU-GEMM, the most immature progenitors. In the case of hematopoietic progenitors, IL-1 most likely functions as a proliferation factor. By flow cytometric analysis, we also observed IL-1β increases in the number of Mac1$^+$ cells in AGM organ cultures in a dose dependent manner. The increase in Mac1$^+$ cells, together with the increase in CFU-M progenitors, indicates that IL-1β enhances AGM myelopoiesis, particularly at high doses. This effect of IL-1 on the myeloid compartment is consistent with *in vitro* and *in vivo* studies in adults, in which it was shown that IL-1 increases the number of myeloid progenitors [12,20,22].

It is unlikely that the major effects of IL-1 on AGM hematopoiesis are direct. Flow cytometric analyses reveal only a small number of IL-1R$^+$CD45$^+$ and IL-1R$^+$c-kit$^+$ AGM and these hematopoietic cells express low levels of IL-1R as compared to AGM endothelial or mesenchymal cells. Rather, we suggest that IL-1 mediates it effects via the AGM microenvironment by the production of proliferation/differentiation factors. Extensive RT-PCR analysis for the gene expression changes of several cytokines and other hematopoietic regulators in AGM tissues after organ culture did not reveal a clear change in the expression of *Kitl* (SCF), *Il6* or *Tgfb* superfamily factors which could contribute to the physiological effects we observed (not shown). However, in the UG26-1B6 stromal cell line, we were able to detect changes in gene expression of hematopoietic regulators induced by IL-1, showing that IL-1 affects the hematopoietic microenvironment. The fact that all AGM stromal cell lines express

IL-1RI and that high IL-1RI expression levels correlate with better HSC support capacity underline this notion [36]. However, our studies do not rule out the possibility that IL-1 also directly regulates a few AGM hematopoietic cells. It is noteworthy to mention that on primary hematopoietic cells the IL-1RI is expressed at very low levels. We have enhanced the IL-1RI signal in our immunostaining with biotin-streptavidin staining which also increases background. In our flow cytometric studies we have used a directly conjugated fluorescent anti-IL-1RI antibody. Thus, when analysing subpopulations of cells with additional antibodies, it is highly likely that we missed some AGM (hematopoietic) cells that express only low levels of the IL-1RI. Future studies will test whether IL-1 signalling of AGM hematopoietic cells may be (direct or) autocrine and important for the upregulated expression of IL-1.

Possible roles for IL-1 in hematopoietic cell migration and homing in the embryo

In the adult, IL-1 has also been implicated in the mobilization of HSCs from the adult BM to the peripheral blood [13]. IL-1 is well known for its effects on the vascular endothelium by upregulating cell adhesion molecules, such as ICAM, for recruitment of leukocytic cells and for inducing the expression of chemoattractant cytokines, such as IL-8 in epithelial and fibroblast cells [7,31]. Moreover, it enhances the adhesion of $CD34^+$ bone marrow cells to bone marrow vascular endothelial cells [44]. During mid-gestation, while the AGM allows for the generation, expansion and maintenance of HSCs, colonization of hematopoietic tissue rudiments (fetal liver) by newly formed hematopoietic cells must

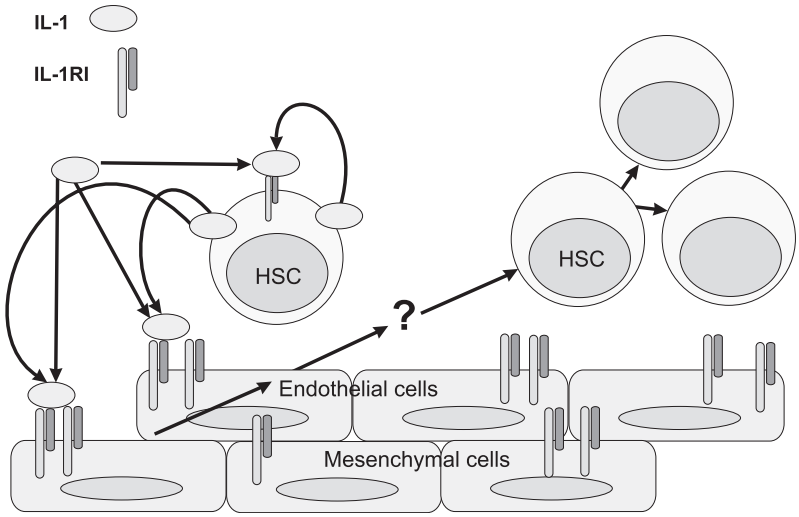

Figure 6. Model of IL-1 related interactions in the E11 mouse AGM region
The IL-1RI is expressed on endothelial, mesenchymal and at lower levels on some HSCs. Receptor expression appears to be stable. The expression of IL-1 is variable, with high levels being produced by HSCs and other hematopoietic cells. Expression of IL-1 is low or negligible in the endothelial and mesenchymal compartment. IL-1 (from HSCs or other hematopoietic cells) is thought to interact with IL-1RI expressing endothelial and/or mesenchymal cells on the ventral side of the E11 dorsal aorta. IL-1RI signalling results in the induction of unknown factor(s) indicated with a question mark (?) to trigger the growth and/or proliferation of HSCs.

occur for the ongoing development of the adult hematopoietic system. For this reason, the effects of IL-1 on AGM HSC adhesion and migration are interesting. Since others have found that IL-1 induces MMP9 expression in HUVEC and vascular smooth muscle cells [18,28], we examined *Mmp9* expression levels in the AGM. We found that *Mmp9* was upregulated in cultures of AGM explants containing IL-1 (Figure 5B). Thus, further studies should reveal whether *Mmp9* is a direct IL-1 target gene and involved in hematopoietic cell migration in the embryo. In addition, we observed the downregulation of *Cxcl12* (SDF1α and β) in AGM explants cultured in the presence of IL-1 (Figure 5B). Previously, others have found that SDF1α and CXCR4 are required for the establishment of normal bone marrow hematopoiesis but not for normal fetal liver hematopoiesis [1]. However, the role of these molecules in the AGM has not yet been explored.

IL-1 increases AGM hematopoietic stem and progenitor cells.

IL-1 was able to increase the number of hematopoietic $CD45^+$ and progenitor $c\text{-}kit^+$ cells in AGM organ cultures as well as the number of progenitor cells. These increases can be accounted for by increased proliferation or decreased apoptosis. Since we observed that the total number of AGM cells was slightly decreased after the organ cultures in the presence of IL-1β as compared to the control cultures, we examined whether IL-1β affected hematopoietic cell viability. RT-PCR analysis revealed that the anti-apoptotic genes *Bcl2* and *Bcl2l1* (Bcl-x) were downregulated in AGM tissues cultured in the presence of IL-1 (not shown), but in flow cytometric studies with Annexin V staining, we found no consistent effect of IL-1 on the viability of $c\text{-}kit^+$ cells (not shown). Thus, IL-1 does not appear to affect changes in the survival/apoptosis of a majority of hematopoietic progenitors, although IL-1 could be affecting the apoptosis/survival of other AGM cell populations. Nevertheless, we did observe a decrease in long term repopulating HSCs and CFU-GEMM in AGM explants treated with a high dose of IL-1β (10 ng/ml). This loss of function may be due to apoptosis of HSCs or alternatively, IL-1β at high doses may trigger the differentiation of these cells. The fact HSC activity is increased in AGM explants cultured with low dose IL-1β (1 ng/ml) is consistent with reports that low concentrations of IL-1 have a different effect on BM HSCs than higher concentrations [26] and strongly suggests that low dose IL-1 affects the proliferation of HSCs/progenitors and high dose IL-1 induces their differentiation. A comprehensive study of the dose effects of IL-1 on AGM stroma may reveal changes in the regulation of specific molecules relevant to HSC activity and differentiation. Thus, IL-1 regulation of AGM hematopoiesis is both cell context- and dose-dependent.

In conclusion, our data show that IL-1 can act as a regulator of the earliest adult repopulating HSCs in the mouse embryo. Future studies will show how this previously thought of "adult" cytokine acts to stimulate hematopoiesis and regulate the proliferation and/or migration of these cells in the AGM and other rapidly developing tissues of the hematopoietic system.

Acknowledgements

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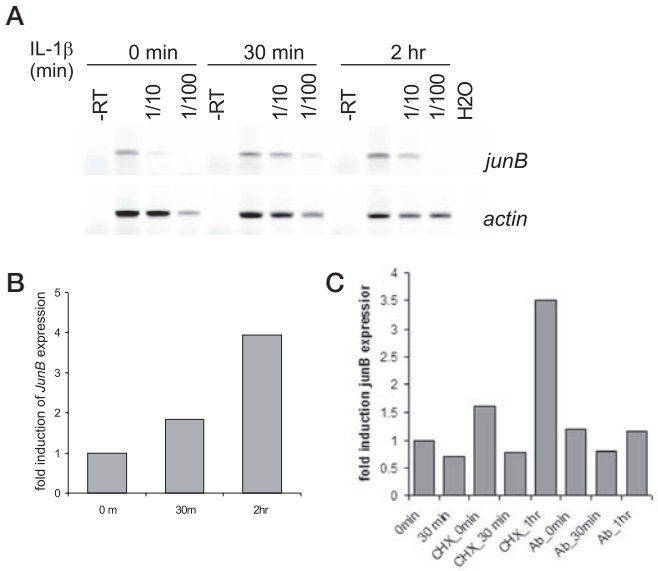
Supplementary Table 1. RT-PCR primer sequences

Primer name	Primer sequence (5'-3')	Product size (bp)	Accession number
β-*actin* for	CCTGAACCCTAAGGCCAACCG	397	X03672
β-*actin* rev	GCTCATAGCTCTTCTCCAGGG		
Cxcr4 for	TGACTTCGAGAGCATTGTGC	518	NM_009911
Cxcr4 rev	TCCTCTACAGTTCTACAGTC		
Csf1 for	GAGAAGACTGATGGTACATCC	636	X05010
Csf1 rev	CTGTACTCTGAGCTGGTCAG		
Csf2 for	CAAAGAGGCAGACCTGAGAC	350	X03020
Csf2 rev	GAAGCTGGATTCAGAGCTGG		
Il1rap for	GAAATAGCCTCAGCTCACACAG	458	NM008364
Il1rap rev	CTCTGTTCCACCTCAGACTC		
Il1r1 for	ACCTCACTTCTCCTGGATCC	300	NM008362
Il1r1 rev	GGGGTACAAAGAACAAGGCG		
Il1r2 for	CCAGCATCATTGGGGTCAAG	435	NM010555
Il1r2 rev	CCTGGTTGTCAGTCCGTAGC		
Irak4 for	CATGACCAGCCGAATCGTGG	282	AF445803
Irak4 rev	CAGACACTGGCTAGCAGCAG		
Junb for	GCAGCTCACACACTGGACTC	296	BC003790
Junb rev	CTTTAGACACGAAGTGCGTG		
Mmp9 for	ATGTGTCCCACTATACCTCC	464	X72794
Mmp9 rev	GACCCAACTTATCCAGACTC		
Sod2 for	CATCCACTTCGAGCAGAAGG	442	X06683
Sod2 rev	AGTCTGAGACTTCAGACCAC		
Kitl for	CTTTGGTGAACTTTCATGTG	1042	NM_013598
Kitl rev	TGTGGATCACTCCTAAGCCC		
Cxcl12 for	ACCAGTCAGCCTGAGCTACC	928	L12029
Cxcl12a rev	GGAAGTCAGATGAAGCATGC		
Cxcl12b rev	CGATCATGAGTGAGAAGGAC	873	L12030
Map3k7ip2 for	TGCTGGTGCACCAGAAGGCCAGGATG	686	BC004813
Map3k7ip2 rev	GTCTCTCAGGCCTTTCAGGTG		
Map3k7 for	GTGGTACATTACAGAGGGAC	412	BC006665
Map3k7 rev	CCGATAGCTCAGCTCAAGCC		
Traf6 for	CATTTATGCACCTGGAAGCC	423	NM_009424
Traf6 rev	CCCATGGAAGCACAGTGAAG		



Supplementary Figure 1. Control optimisation experiments examining the effects of IL-1β stimulation on 3T3 fibroblast cells

(A) 3T3 fibroblasts were stimulated with IL-1β (10 ng/ml) for 0 minutes, 30 minutes or 2 hours and RNA was isolated and used for cDNA synthesis. Serial dilutions of the cDNA were used for semi-quantitative RT-PCR and analysis was performed with Typhoon and ImageQuant software. IL-1β stimulation of 3T3 fibroblasts results in increased expression of the IL-1β target gene *Junb* as determined by semi-quantitative RT-PCR. **(B)** Fold induction of *Junb* expression induced by IL-1β stimulation in 3T3 cells. Results calculated from RT-PCR values. **(C)** Schematic presentation of the RT-PCR results (fold induction) show that IL-1β induced *Junb* gene induction in 3T3 fibroblasts can be blocked with an IL-1β blocking antibody (Ab) and that cyclohexamide (CHX) does not interfere with the induction of the immediate early *Junb* target gene.

Chapter 4

Interleukin-1 increases fetal liver hematopoietic progenitor cells in the mid-gestation mouse embryo

Claudia Orelio, Marian Peeters, Esther Haak, Karin van der Horn and Elaine Dzierzak

The information of this chapter is in preparation for publication

Abstract

Background and objective. Hematopoietic progenitors are generated in the yolk sac and aorta-gonad-mesonephros (AGM) region in mouse mid-gestation. At embryonic day 10.5 (E10.5) the first hematopoietic stem cells (HSCs) emerge in the AGM. Subsequently, hematopoietic progenitors and stem cells can be detected in the fetal liver (FL). The FL is a potent hematopoietic site, which plays an important role in the expansion and differentiation of hematopoietic progenitors and HSCs. However, little is known concerning the regulation of HSCs in the FL. Particularly, the role of cytokines such as Interleukin-1 (IL-1) in the regulation of HSCs in the embryo has been largely unexplored. Recently, we observed that the adult pro-inflammatory cytokine IL-1 is involved in regulating hematopoietic progenitor and HSC activity in the AGM region. Therefore, we set out to investigate whether IL-1 also plays a role in regulating FL HSCs. *Methods*. First, we examined whether the ligand IL-1 and its receptor IL-1RI are expressed in the fetal liver. Next, we examined the effect of IL-1 on hematopoietic progenitor cells and HSCs by FACS analysis and transplantation studies. *Results*. We show that FL hematopoietic progenitor cells express the IL-1RI and more importantly that IL-1 increases FL hematopoiesis, progenitor cell activity and promotes hematopoietic cell survival. However, unlike its effect on the AGM HSCs, IL-1 does not affect FL HSC activity. *Conclusions*. IL-1 is a regulator of FL hematopoiesis and hematopoietic progenitor cells, but not of FL HSCs and thus IL-1 acts differentially on HSCs in distinct microenvironments.

Introduction

The adult hematopoietic system consists of at least ten distinct blood cell lineages that are produced through the differentiation of hematopoietic stem cells (HSCs) and many intermediate progenitor cells. In the adult, HSCs reside in the bone marrow. In the developing mouse, the first adult long-term repopulating HSCs are found at embryonic day 10.5 (E10.5) in the intra-embryonic aorta-gonad-mesonephros (AGM) region [16,20]. Slightly later, from E11 onwards these HSCs are detected in other hematopoietic tissues, including the fetal liver (FL) [13,19]. The FL commences its role as an important embryonic hematopoietic organ at late E9 [10,12]. Between E11 and E16 HSC numbers are increased dramatically in the FL and subsequently remain constant until birth [8,13,19], when the bone marrow takes over as the HSC niche through the adult stages of life. Besides the expansion of HSCs, the FL also plays a crucial role in erythropoiesis and hematopoietic progenitor cell expansion [28,33,34].

In contrast to the AGM region, which harbours a microenvironment suitable for the generation of HSCs, the FL does not generate HSCs *de novo*, but is thought to be seeded with HSCs from other embryonic sites (i.e. the AGM and/or the yolk sac) [3,10,12,13,18]. Indeed, β1-integrin deficient embryos contain hematopoietic stem/progenitors in the yolk sac and circulation, but none are found in the FL or BM suggesting impaired migration and colonization of these hematopoietic tissues [29]. Subsequent to colonization by hematopoietic cells the FL provides an excellent *in vivo* environment for HSC expansion, as demonstrated by the dramatic increase in HSC activity during mid-gestation [8,19]. Also, several FL stromal cell lines have been shown to maintain and/or expand HSCs *in vitro,* further indicating that the FL contains an HSC supportive microenvironment [17,23,24].

Despite the fact that the FL is a pivotal organ for HSC expansion during development, little is known about the cytokines and growth factors that affect hematopoiesis, and more specifically hematopoietic progenitors and HSCs, within this tissue. Previously, we have shown that the Interleukin-1 (IL-1) signalling component TAB2 is expressed in the AGM region at the time of HSC appearance [26]. Additionally, we observed that several IL-1 receptor/signalling components are expressed in the midgestation AGM and that IL-1 increased AGM HSC activity and hematopoiesis (Orelio et al., Chapter 3). Since TAB2 is expressed in the FL, IL-1 may also regulate FL hematopoiesis. It is well documented that the pro-inflammatory cytokine IL-1 regulates adult hematopoiesis and plays a role in a number of diseases, including autoimmune diseases and leukaemia [6]. Besides regulating mature, differentiated hematopoietic cells, functional studies show that IL-1 regulates adult BM HSCs by providing these cells with differentiation and/or proliferation and radio-protective signals [2,9,11,21,25]. Since IL-1 and the IL-1 receptor are expressed by BM hematopoietic stem/progenitor cells, it has been suggested that IL-1 acts directly on these immature cells at the base of the hematopoietic hierarchy [15,35,36].

In this study we examine a role for IL-1 in FL hematopoiesis. We show that the IL-1 receptor type 1 (IL-1RI) is expressed on FL hematopoietic (progenitor) cells and that its ligand IL-1 is also expressed in this tissue. The addition of exogenous IL-1 to FL explants increases hematopoietic progenitor activity and the overall number of hematopoietic cells. In contrast to our findings that IL-1 increases AGM HSC activity, we observed no alteration in HSC activity in the FL. Hence, while IL-1 appears to be a

general activator of hematopoiesis throughout development, these FL data underscore developmental differences in IL-1 mediated HSC activity in mid-gestation hematopoietic sites.

Material and Methods

Embryo generation and cell culture

Animals were housed according to institutional guidelines, with free access to water and food. Animal procedures were carried out in compliance with the Standards for Humane Care and Use of Laboratory Animals. To generate embryos, matings were set up between (C57/BL10xCBA)F_1 females and males. For the transplantation studies transgenic males (Ly6A-GFP and Ln72 human β-globin) were used for generating embryos. The day of the vaginal plug was counted as day 0. Pregnant mice were sacrificed by cervical dislocation and livers were dissected as described. For obtaining single cell suspensions of embryonic material, tissues were collagenase treated (0.125% in PBS/ 10% FCS) for 1 hour at 37°C.

RNA isolation, cDNA synthesis and RT-PCR analysis

Total RNA was isolated with TRIZOL (Gibco/ Life Technologies) according to manufacturer's instructions and DNAse treated with RQ1 RNase free DNAse (Promega). For cDNA synthesis 1-5 μg of total RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen/ Life Technologies) according to manufacturer's instructions.

PCR reactions were performed in 50 μl with 1U Amplitaq (PerkinElmer) and provided buffer, 100 ng of each primer, 1mM dNTPs, and 2 μl of cDNA. PCR cycles as follows: 5 minutes 92°C, 27-40x (40 sec 92°C, 40 sec 58°C, 1 minute 72°C), 7 minutes 72°C. PCR primers are listed in Supplementary Table 1.

Immunohistochemistry

Embryos were snap-frozen in TissueTek (Sakura) and 7-10μM cryosections generated. Sections were fixed in 2% paraformaldehyde/PBS and endogenous peroxidase activity was blocked with 1.2% H_2O_2/ MeOH. Sections were blocked with TSA blocking buffer and incubated with IL-RI antibody (clone 12A6; BD biosciences) overnight at 4°C. Subsequently, sections were incubated with α-rat-biotin antibody and streptavidin-HRP (DAKO) in combination with TSA biotin system (PerkinElmer) according to provided instructions. Staining was visualized with DAB chromogen (Sigma) and sections counterstained with hematoxylin. Sections were embedded with Entallan (Merck). Pictures were taken with an Olympus BX40 microscope.

Organ cultures and *in vivo* transplantation assays for HSC activity.

E11 liver tissues (marked with GFP or human β-globin) were dissected and 2-3 day organ cultures were performed as described previously in the presence of 0, 1 or 10 ng/ml IL-1β (TebuBio). To inhibit

IL-1 signalling, 50 ng/ml IL-1Ra (R&D systems) or 100 ng/ml IL-1α blocking antibody (R&D systems) was added to the cultures.

After the culture single cell suspensions were obtained and different cell dilutions (measured as embryo equivalents) were injected intravenously together with non-marked $2x10^5$ spleen cells into 9.5 Gy irradiated (C57BL/10xCBA)F_1 recipient mice. For the expression analysis, cultured tissues were homogenized in TRIZOL and processed as described. For FACS analysis, single cell suspensions were obtained and processed as described.

Repopulation was assayed at 1 and 4 months post-transplantation by donor specific PCR (human β-globin or GFP) on peripheral blood DNA as described previously [4,5]. Only mice with >10% engraftment were considered repopulated. For multi-lineage repopulation analysis DNA was isolated from spleen, thymus, bone marrow and peripheral blood or from FACS-sorted cells from these tissues and assayed for donor contribution by PCR.

FACS analysis

Single cell suspensions were stained with IL-1RI-PE (clone 35F5, Becton Dickinson) antibody on ice for 30 minutes. Cells were co-stained with FITC labelled antibodies for c-kit (CD117), Mac-1 (CD11b) or CD45, CD31 (Pharmingen). Dead cells were excluded by 7AAD (Molecular Probes) and FACS analysis was performed on FACScan (Becton Dickinson).

Intracellular FACS

Single cell suspensions were made in the presence of GolgiPlug (Becton Dickinson) of (fresh or cultured) fetal liver tissues as described. Cells were stained with extracellular markers as described and cells were fixed and permeabilized with Cytofix/Cytoperm solution (Becton Dickinson). Thereafter cells were stained with a PE-conjugated IL-1α antibody (clone ALF161, Becton Dickinson) and analysed with a FACScan (Becton Dickinson).

Statistical Analysis

Data are expressed as mean ± SEM. Differences were considered to be significant at $p<0.05$ as analysed with a Student's t-test.

Results

FL hematopoietic cells express IL-1RI

To examine whether IL-1 could play a role in the expansion of hematopoietic progenitors and HSCs in the FL, we tested for the expression of IL-1RI on FL cells by flow cytometric analysis. We observed that on average 5.1% of E11.5 and 3.4% of E12.5 FL cells (Table 1) express the IL-1RI. During this one-day period the total number of FL cells increases 7.6 fold ($4.9x10^5 \pm 1.0x10^5$ at E11.5 and $37x10^5 \pm 11x10^5$

at E12.5). Hence, the absolute number of FL IL-1RI$^+$ cells increases 5.2 fold from E11.5 ($0.24x10^5$) to E12.5 ($1.26x10^5$).

Multi-parameter flow cytometry with the pan-hematopoietic marker CD45 revealed that E11.5 and E12.5 FL contains on average 1.1% and 0.3% IL-1RI$^+$CD45$^+$ cells respectively (Figure 1A and Table 1), indicating that 9 to 23% of the IL-1RI$^+$ population is hematopoietic. Moreover, we observed that 1.3% and 0.7% of E11.5 and E12.5 FL cells respectively are IL-1RI$^+$c-kit$^+$, suggesting that most of the IL-1RI$^+$ hematopoietic cells are progenitor/stem cells. This is supported by flow cytometric analysis demonstrating that some FL IL-1RI$^+$ cells express CD31 and Mac1 (not shown), markers of FL HSCs and myeloid cells [19,31].

In addition to the expression of the IL-1RI, we observed by immunostaining of E12.5 FL that the ligand IL-1β is expressed in small patches of cells (Figure 1B) and flow cytometric analysis revealed that 0.04-0.11% of E11.5 FL cells express the IL-1α ligand (data not shown). Thus, both IL-1 expressing cells and putative IL-1 responsive cells are present in the fetal liver.

IL-1 increases the number of FL hematopoietic cells

To determine whether IL-1 affects the number of hematopoietic (stem/progenitor) cells in the FL, we performed *ex vivo* organ cultures in the presence or absence of IL-1 followed by flow cytometric analysis. As shown in Figure 2A, IL-1 increases the percentage of FL CD45$^+$, c-kit$^+$ and Mac1$^+$ cells in a dose-dependent manner. With the addition of 1 ng/ml IL-1, the percentage increases averaged 1.4 to 1.6 fold as compared to control cultures. Addition of 10 ng/ml IL-1 significantly increases the percentages of these populations with on average 1.7 to 2.3 fold. The absolute number of CD45$^+$, c-kit$^+$ and Mac-1$^+$ cells was similarly increased (Supplementary Figure 1B), as IL-1 does not affect total cell numbers in cultured liver explants (Supplementary Figure 1A).

To address whether the increase in FL hematopoietic (stem/progenitor) cells was specifically induced by IL-1, we blocked IL-1 signalling in these organ cultures. We used either a natural IL-1 receptor antagonist (IL-1Ra), which binds to the IL-1RI but does not evoke receptor signalling, or an IL-1α specific blocking antibody. Both IL-1Ra (Figure 2B) and the IL-1α blocking antibody (not shown) significantly decreased the percentage of CD45$^+$ and c-kit$^+$ cells in the cultures by an average

Table 1. Percentages of IL-1R$^+$ hematopoietic cells in E11 and E12 fetal liver tissues

	E11-11.5 liver		**E12-12.5 liver**	
	mean	(range)	Mean	(range)
IL-1RI$^+$CD45$^-$	4.0	3.5-4.5	3.2	1.8-4.2
IL-1RI$^+$CD45$^+$	1.1	0.7-1.5	0.3	0.1-0.6
IL-1RI$^-$CD45$^+$	11.6	10.1-15.3	6.6	5.6-7.0
IL-1RI$^+$c-kit$^-$	3.5	3.0-4.3	2.5	1.7-2.8
IL-1RI$^+$c-kit$^+$	1.3	1.1-1.7	0.7	0.2-1.1
IL-1RI$^-$c-kit$^+$	22.9	15.3-39.4	12.5	9.9-14.4

These data are combined from several independent experiments in which several FLs from age-matched littermates were pooled for IL-1RI flow cytometric analysis on hematopoietic (progenitor) cells. E11-E11.5 FL (n=5) and E12-E12.5 FL (n=4).

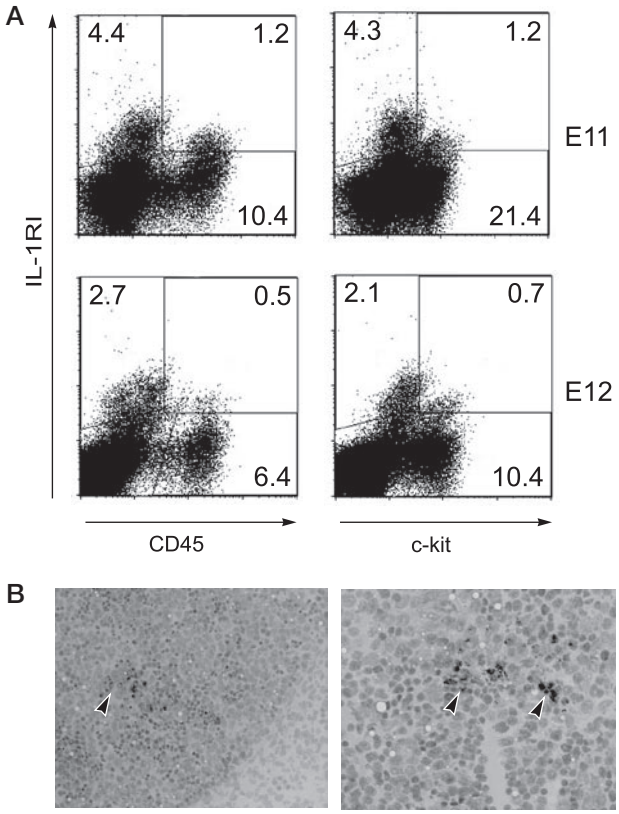


Figure 1. IL-1 and the IL-1 Receptor are expressed in the mid-gestation fetal liver
(A) Multiparameter flow cytrometric plots of E11 and E12 fetal liver cells showing IL-1RI, CD45 (hematopoietic cell marker) and c-kit (hematopoietic stem/progenitor marker) expression. Percentages of single positive and double positive cells are indicated in the quadrants. The percentage of IL-1RI$^+$ cells in the E11 FL is slightly higher than in the E12 FL. Approximately 80,000 events are shown. **(B)** Immunohistochemistry for IL-1β protein expression on transversal E12 embryo trunk sections. IL-1β expression can be detected in cell clusters indicated by the arrowheads. Original magnification 200x (left) and 400x (right) (colour print, see back side cover).

of 0.8 and 0.7 fold. Together these data show that IL-1 allows specific expansion of FL hematopoietic (stem/progenitor) cells and strongly suggest that IL-1 signalling plays a physiological role in regulating FL hematopoiesis.

IL-1 affects on FL gene expression and apoptosis

The IL-1 mediated increase in FL hematopoietic cells in organ cultures may be a consequence of gene expression changes that could contribute to the expansion of hematopoietic cells or their resistance to apoptosis. Therefore, we performed RT-PCR analysis for IL-1 and other cytokine signalling pathway genes (Figure 3A/B) and cell survival-related genes (Figure 3C) on RNA from FL explants cultured in the absence or presence of IL-1. The gene expression levels of IL-1 receptor components and the IL-1 ligands

did not change in the presence IL-1. Also, the gene expression levels of the *Csf1* (M-CSF), *Csf3* (G-CSF) and *Kitl* (SCF) genes were unchanged. Interestingly, while the expression levels of the anti-apoptotic genes, *Bcl2* or *Slugh* were unaffected by IL-1, another anti-apoptotic gene *Bcl2l1* (Bcl-x) was upregulated. Levels of the pro-apoptotic *Bax* and *Bim* genes were not affected by IL-1. Thus, IL-1 could be influencing the viability of FL hematopoietic cells through the modulation of apoptotic pathways.

To test this, flow cytometric analysis for the pre-apoptotic marker, AnnexinV, in combination with CD45 and c-kit was performed on FL explants After 2 days of culture, the percentage of AnnexinV$^+$ cells in the CD45$^+$ FL cell population was significantly decreased in the presence of IL-1 (Figure 4A/B). The percentage of apoptotic cells in the c-kit$^+$ FL cell population was similarly decreased in the presence of IL-1 (not shown). Thus, IL-1 affects FL cell numbers by promoting hematopoietic cell survival.

IL-1 increases FL hematopoietic progenitor activity

To test the effect of IL-1 on immature FL cells (i.e. short-term repopulating hematopoietic progenitors and long-term repopulating HSCs), we performed *in vivo* transplantation experiments. Cells from E11.5 liver explants cultured in the absence or presence of 1 ng/ml or 10 ng/ml IL-1β were injected into irradiated adult mice and examined at 1 (short-term) and 4 (long-term) months post transplantation. Recipients were examined by peripheral blood DNA PCR for the presence of the donor cell genetic marker (human beta-globin). As shown in Figure 5A, the low dose of IL-1β (1 ng/ml) but not the high dose (10 ng/ml) increased the percentage of repopulated mice at 1 month post-transplantation, indicating that IL-1β increases short-term repopulating hematopoietic progenitor activity in the FL. In contrast, at 4 months post-transplantation, neither dose of IL-1 affected long-term repopulating HSC activity in the FL. The same percentage of multilineage, high-level engrafted recipients were found as with control FL cultured in the absence of IL-1 (Figure 5B). Thus, IL-1 increases short-term FL hematopoietic progenitor activity, but has no positive or negative effect on long-term FL HSC activity.

Discussion

We have shown here that the well-known inflammatory cytokine IL-1 plays a role in the regulation of hematopoietic cells and hematopoietic progenitors in the FL during mid-gestational development. Both the ligand IL-1 and the IL-1 receptor are expressed in the E11-E12 FL. Moreover, exogenous IL-1 induces an increase in hematopoietic cell numbers as well as short-term repopulating hematopoietic progenitors in FL explants. Although the FL efficiently expands HSCs during a short window of developmental time (E11 to E16), it appears that IL-1 does not play a role in this process. No IL-1 mediated increase in FL HSCs was found by the *in vivo* long-term repopulating assay. Also, no decrease in FL HSC activity was found, suggesting that IL-1 has neither a positive nor a negative effect on FL HSCs. Thus, within the FL our data strongly suggest that IL-1 mainly acts on hematopoietic progenitors. It could act either directly on IL-1RI expressing hematopoietic progenitors or indirectly on the cells of the FL microenvironment that in turn affect hematopoietic progenitors.

Developmental differences in IL-1 mediated hematopoietic cell regulation

Recently, we showed that IL-1 plays a role in regulating mid-gestation AGM hematopoietic cells and that the addition of exogenous IL-1 increases AGM HSC and progenitor activity. Compared to the AGM, the levels of IL-1RI expression, as well as the percentages of IL-1RI$^+$ cells, are higher in the FL. Our finding of the restricted effect of IL-1 on FL hematopoietic progenitor activity suggests a differential role for IL-1 signalling in these two embryonic hematopoietic microenvironments.

Similar to the AGM region, we observed in the FL that the IL-1RI is not exclusively expressed on CD45$^+$ hematopoietic cells. Approximately 60-90% of FL IL-1RI$^+$ cells do not express the CD45 or

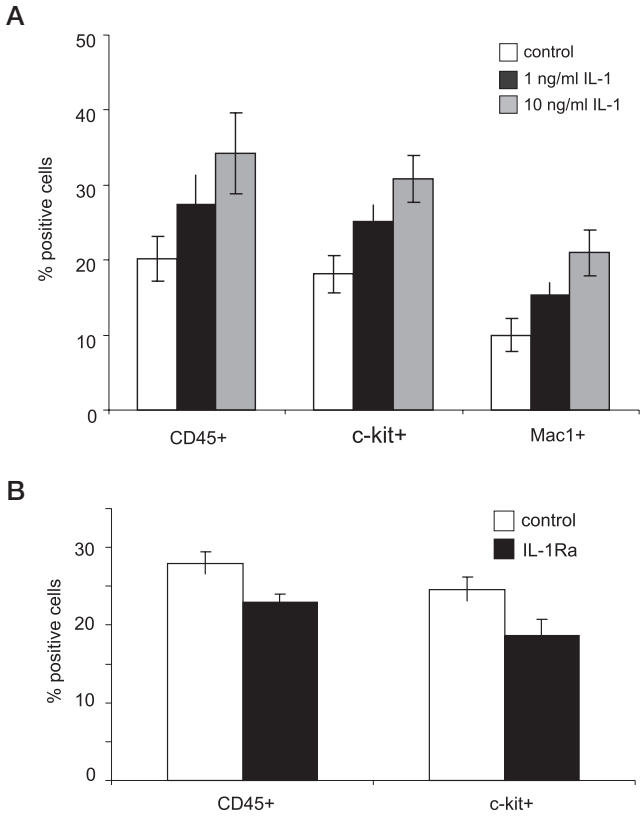


Figure 2. IL-1β increases hematopoietic progenitor cells in the E11 FL
E11 FL tissues were cultured for 3 days as explants in the absence or presence (1 ng/ml or 10 ng/ml) of IL-1β or IL-1R antagonist (IL-1Ra). After this culture period, single cell suspensions were generated and the percentage of hematopoietic (CD45$^+$), or hematopoietic progenitor/stem cells (c-kit$^+$ or Mac1$^+$) was determined by flow cytometric analysis. **(A)** IL-1β increases, in a dose-dependent manner, the percentage of CD45$^+$, c-kit$^+$ and Mac1$^+$ liver cells after organ culture. The bars indicate the average percentage of marker positive cells from 5 independent experiments and the error bar indicates the SEM. **(B)** IL-1R antagonist (IL-1Ra) decreases the percentage of CD45$^+$ and c-kit$^+$ FL cells after organ culture. The bars indicate the average percentage of marker positive cells from 3 independent experiments and the error bars indicate the SEM.

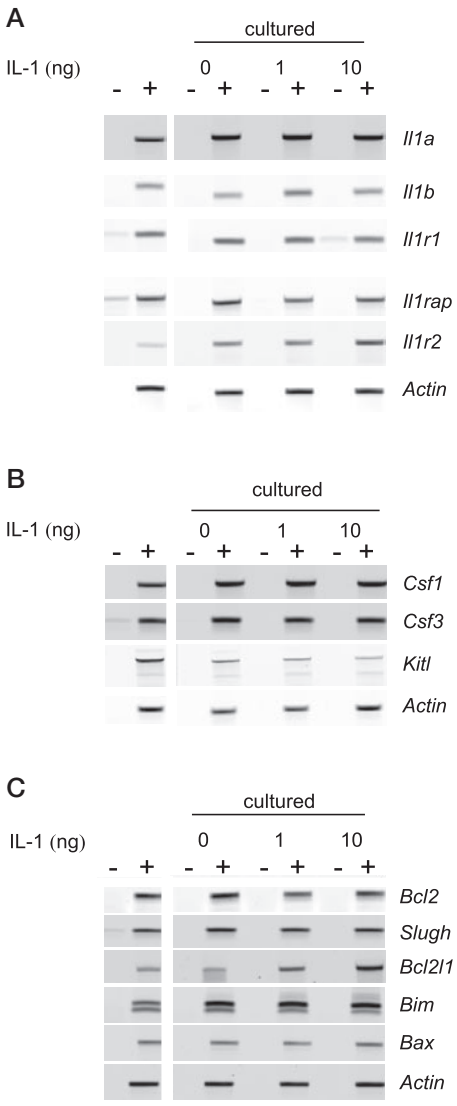


Figure 3. IL-1 effects on gene expression levels of hematopoietic growth factors in FL organ cultures
E11.5 FL tissues were cultured for 3 days in the presence or absence of 1 ng/ml or 10 ng/ml IL-1β. After the culture, RNA was isolated and RT-PCR analysis was performed for several hematopoietic-related genes. **(A)** Gene expression analysis for the ligands IL-1α and IL-1β (*Il1a and Il1b*), subunits to the IL-1 receptor IL-1RI and IL-1R accessory protein (*Il1r1 and Il1rap*), and the decoy IL-1RII (*Il1r2*). No changes in the expression levels of these genes were detected. **(B)** Gene expression analysis for the hematopoietic growth factors M-CSF (*Csf1*), G-CSF (*Csf3*) and SCF (*Kitl*). No changes in the expression levels of these genes were detected. **(C)** Gene expression analysis for the anti-apoptotic genes Bcl-2 (*Bcl2*) and *Slugh* and the pro-apoptotic *Bax* and *Bim* genes. Only the anti-apoptotic gene Bcl-x (*Bcl2l1*) is upregulated in FL in the presence of IL-1. Expression of the housekeeping gene *Actin* was used as a normalization control for the samples.

c-kit hematopoietic markers. This is in contrast to our findings in the adult BM, in which more than 99% of the IL-RI$^+$ cells are CD45$^+$ (unpublished data). Interestingly, by RT-PCR and FACS analysis we observed IL-1RI and IL-1RAcP expression in several AGM and FL derived stromal cells. Moreover, immunostainings of FL and AGM tissues localize IL-1RI expression to both hematopoietic and mesenchymal cell regions. Despite the almost exclusive expression of IL-1RI on adult BM hematopoietic cells, the adult BM stromal cell line FBMD-1 also expresses IL-1RI and IL-1RacP (Orelio et al. Chapter 3). Thus, the expression of IL-1RI on both hematopoietic and non-hematopoietic cells suggests that IL-1 plays a role in regulating hematopoiesis in both a direct and an indirect manner. And while

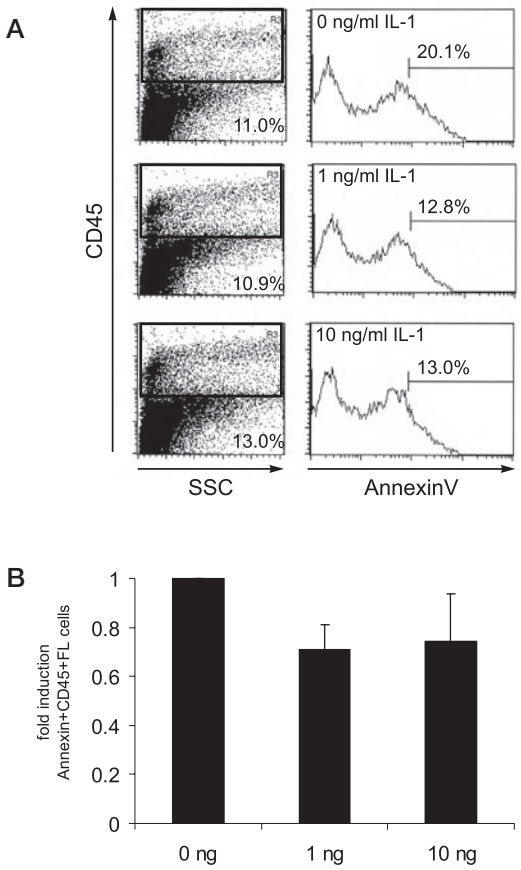


Figure 4. IL-1 affects apoptosis in the E11 liver

(A) Representative flow cytometric plots showing the amount of apoptotic hematopoietic cells in FL explants after 2 days of culture. The percentages of CD45$^+$ cells are shown in the left panels and the percentages of AnnexinV$^+$ cells within the CD45$^+$ population of FL cells are shown in the right panels. **(B)** Bar graph indicating the fold induction of the average percentage of AnnexinV$^+$ (apoptotic) cells within the hematopoietic (CD45$^+$) fetal liver population after 2 days of organ culture in the presence or absence of IL-1. Error bars indicate the SEM. (n=3)

IL-1 may in a limited way act on the adult BM microenvironment (1% of the IL-R1^{+} cells are CD45^{-}), the high percentages of IL-1RI^{+} non-hematopoietic cells in FL and AGM (Orelio et al. Chapter 3) strongly suggest that these embryonic microenvironments are responsive to IL-1 and consequently affect hematopoiesis. These observations support the notion of differential changes in the requirements and interactions within the tissue-specific microenvironments of the hematopoietic system during the various stages of ontogeny.

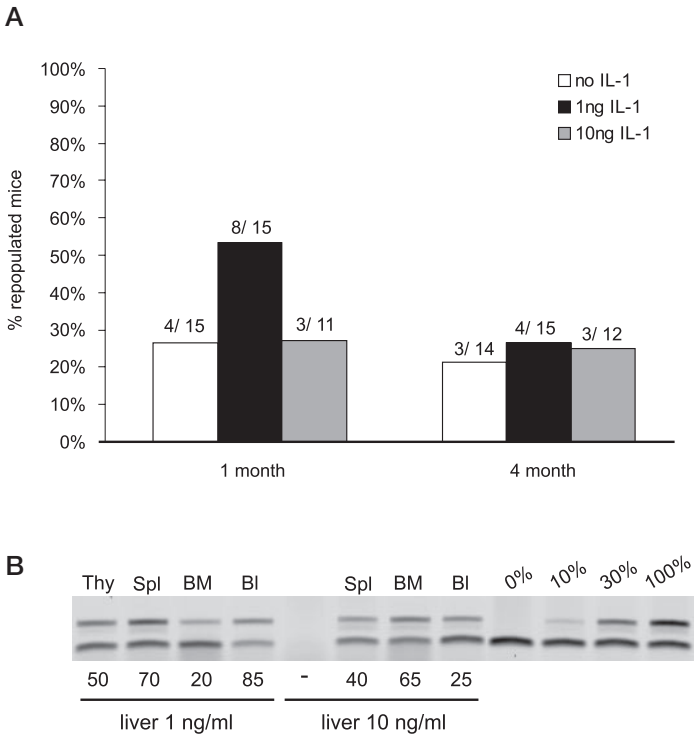


Figure 5. IL-1β increases short-term, but not long-term HSC activity in FL tissues
E11.5 FL tissues were cultured in the absence or presence of 1 ng/ml or 10 ng/ml IL-1β. Single cell suspensions were obtained and injected into lethally irradiated recipients. One and four months post-transplantation the peripheral blood was tested for donor contribution to the hematopoietic system by semi-quantitative PCR. **(A)** Combined results from 7 independent experiments in which 1 embryo equivalent of cultured FL cells were injected per recipient. The bar indicates the percentage of repopulated mice per condition after 1 and 4 month post-transplantation. The numbers above the bars indicate the number of repopulated recipients out of the total number of injected recipients. Only mice with >10% engraftment were considered repopulated and multilineage repopulation was confirmed in several recipients. **(B)** Multilineage analysis for 2 recipients that were injected with FL cells cultured in the presence of 1 ng/ml or 10 ng/ml of IL-1. Genomic DNA was isolated from several hematopoietic tissues and tested for donor hematopoietic contribution by semi-quantitative PCR. All tissues analysed show high levels of donor contribution, confirming that IL-1 does not compromise the ability of the FL HSCs to allow multilineage reconstitution of the recipient.

A role for IL-1 in regulating apoptosis of FL hematopoietic cells

Our finding that IL-1 increases the number of hematopoietic (progenitor) cells in FL organ cultures in a dose-dependent manner suggests that IL-1 may influence cell survival. Previously others and we have shown that apoptosis plays a role in regulating HSCs in the AGM, FL and BM [7,22,27]. Therefore, we tested whether IL-1 might be affecting apoptosis-related processes in these embryonic tissues. In the AGM no clear effect on the expression of apoptosis-related genes was detected (unpublished data). However, IL-1 did increase the expression level of the anti-apoptotic Bcl-x gene in FL explants and decreased apoptosis in the FL hematopoietic (progenitor) population. Interestingly, other studies have shown that mice deficient in IL-1R signalling components, such as TAB2 and NFκB pathway components (i.e. p65 NFκB and IKK) are embryonic lethal, due to severe FL degeneration caused by apoptosis [1,14,30,32]. Taken together, these results suggest that IL-1 receptor signalling contributes to maintaining the balance between the life and death of FL cells. This is further supported by many previous studies of *in vivo* IL-1 administration in which IL-1 has been shown to provide radioprotection. Hence, our studies demonstrate that the survival properties of IL-1 are most likely related to its affect on hematopoietic progenitors and not a direct affect on HSCs.

In conclusion, we have shown here that the pro-inflammatory cytokine IL-1 and its receptor are expressed in the mid-gestation fetal liver and that IL-1 increases hematopoietic (progenitor) cell numbers and activity. One of the possible mechanism by which IL-1 increases these hematopoietic progenitors is via decreasing apoptosis in these cell populations. Thus this study reveals an exciting new role for an adult cytokine in regulating the immature hematopoietic cells during embryonic development.

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Supplementary Table 1. RT-PCR primer sequences

Primer name	Primer sequence (5'-3')	Product size (bp)	Accession number
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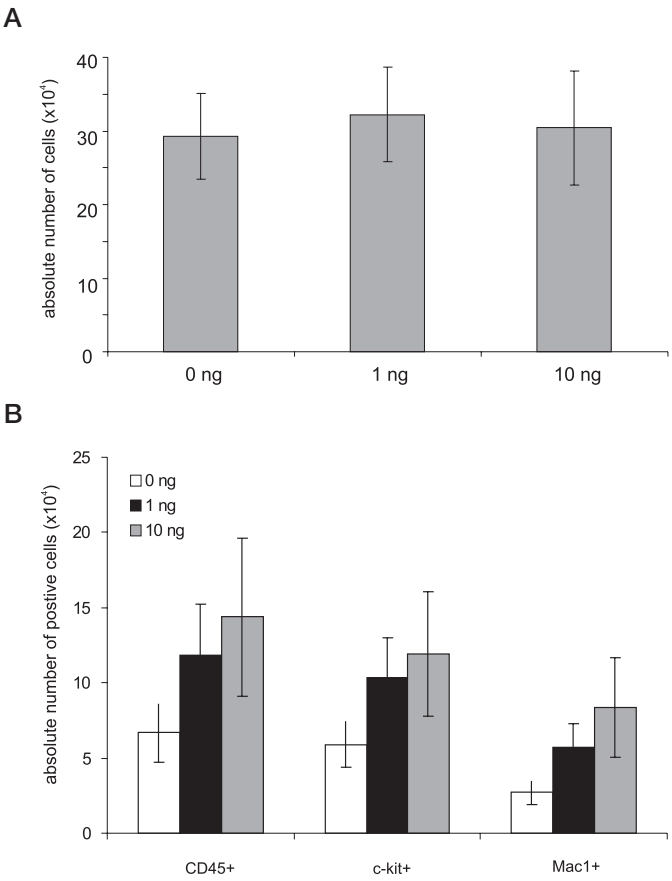
β-*actin* rev	GCTCATAGCTCTTCTCCAGGG		
Bcl2 for	GCACAGATGTCCAGTCAGCTG	268	NM_009741
Bcl2 rev	GCCATATAGTTCCACAAAGGC		
Bax for	CCACCAGCTCTGAACAGATC	694	NM_007527
Bax rev	GACCTGAGGTTTATTGGCGC		
Bcl2l1 for	GGCGATGAGTTTGAACTGCG	916	NM_009743
Bcl2l1 rev	CCTCACTCAATGGCTCTTGG		
Bcl2l11 (Bim) for	GAGAAGGTGGACAATTGCAG	549	AF032459
Bcl2l11 (Bim) rev	CAATGCCTTCTCCATACCAG		
Csf1 for	GAGAAGACTGATGGTACATCC	636	X05010
Csf1 rev	CTGTACTCTGAGCTGGTCAG		
Csf2 for	CAAAGAGGCAGACCTGAGAC	350	X03020
Csf2 rev	GAAGCTGGATTCAGAGCTGG		
Il1a for	GATTCACAACTGTTCGTGAG	741	X01450
Il1a rev	CACTGGAGTAAAACCCACTG		
Il1b for	CCTGTGTAATGAAAGACGGC	334	NM_008361
Il1b rev	GGAGATTGAGCTGTCTGCTC		
Il1rap for	GAAATAGCCTCAGCTCACACAG	458	NM008364
Il1rap rev	CTCTGTTCCACCTCAGACTC		
Il1r1 for	ACCTCACTTCTCCTGGATCC	300	NM008362
Il1r1 rev	GGGGTACAAAGAACAAGGCG		
Il1r2 for	CCAGCATCATTGGGGTCAAG	435	NM010555
Il1r2 rev	CCTGGTTGTCAGTCCGTAGC		
Kitl for	CTTTGGTGAACTTTCATGTG	1042	NM_013598
Kitl rev	TGTGGATCACTCCTAAGCCC		
Slugh for	TCTGCCAGACACTATCTCAG	444	MMU79550
Slugh rev	GCTCATCCAACTACACAAGTC		



Supplementary Figure 1. IL-1β increases the number of E11 FL hematopoietic progenitor cells
E11.5 FL tissues were cultured as explants for 3 days in the presence or absence of 1 ng/ml or 10 ng/ml IL-1β. After this culture period, single cell suspensions were generated and **(A)** the absolute of FL cells was determined. **(B)** The absolute of hematopoietic ($CD45^+$) and hematopoietic progenitor/stem cells ($c\text{-}kit^+$ or $Mac1^+$) was determined by flow cytometric analysis. The bars indicate the average number of marker positive cells from 4 independent experiments and the error bar indicates the SEM.

Chapter 5

Runx1 plays a role in the embryonic hematopoietic microenvironment

Esther Haak, Marian Peeters, Claudia Orelio, Karin van der Horn, Fredrik Wallberg, Rob Ploemacher and Elaine Dzierzak

Abstract

During mouse embryonic development, the first adult-repopulating hematopoietic stem cells (HSCs) are autonomously generated in the aorta-gonad-mesonephros (AGM) region. HSC numbers increase between embryonic day 10.5 (E10.5) and E12 in the AGM indicating that this is a unique supportive microenvironment for HSCs. Runx1 is an important hematopoietic transcription factor that appears to act autonomously in HSCs. Mice deficient for *Runx1* die around E12.5 and lack HSCs. In addition to its function in AGM HSCs, the expression of *Runx1* in endothelial and mesenchymal cells of the AGM region suggests that it may play a role in the stromal microenvironment. To study the role of Runx1 within the embryonic microenvironment we generated stromal cell lines from AGM of wild type and *Runx1*-deficient embryos. These stromal cell lines (parental and cloned) were tested for their functional support of HSCs and hematopoietic progenitor cells. In addition, *Runx1* expression in a *Runx1* +/+ stromal cell line downregulated by RNA interference was examined. Our results show that the growth of hematopoietic progenitors and HSCs is dependent upon the levels of *Runx1* expression in the panel of stromal cell lines and support a role for Runx1 in the AGM microenvironment.

Introduction

The establishment of the hematopoietic system during development begins at embryonic day 7.5 (E7.5) in the yolk sac blood islands where primitive erythroid cells are found [24]. Thereafter, a series of hematopoietic progenitors are generated. At E10.5 the first HSCs able to repopulate an adult recipient appear in the aorta-gonad-mesonephros (AGM) region [11]. During development of the hematopoietic system a wide variety of factors regulate the emergence, survival and proliferation of these cells. Extrinsic factors emanating from the cells of the surrounding microenvironment and cell intrinsic factors such as transcription factors are implicated in these processes.

During the 1990s *AML1* was shown to be frequently involved in chromosomal translocations in leukaemia [14]. The mouse homologue of AML1, Runx1 belongs to the family of core binding factors (CBFs), which bind DNA sequences through their Runt-domain. For proper function they bind to a non-DNA binding subunit, CBFβ, forming a heterodimeric transcription factor complex. *Runx1* deficiency in mice leads to a lack of AGM and fetal liver hematopoiesis suggesting that it plays a crucial role in definitive hematopoiesis [19,28,33]. Furthermore, Runx1 has been found to specifically affect the expression of target genes within the erythroid, myeloid and lymphoid cell lineages either repressing or activating their transcription [18]. *Runx1* directed *LacZ* expression within the AGM region was found in the hematopoietic cells within the aorta-lumen, endothelial cells lining the aortic wall and in the para-aortic mesenchyme [16]. In previous studies Cai et al. [2] showed that this transcription factor complex plays an essential role in the appearance of functional hematopoietic stem cells (HSCs) and immature progenitors in the AGM and yolk sac of the mid-gestational embryo. While no HSCs or immature hematopoietic progenitors are found in embryos completely deficient for *Runx1*, the spatial and temporal development of these cells is altered in the presence of half the dose of *Runx1*. These results suggest that Runx1 plays an intrinsic role in the HSCs during development. Whether Runx1 affects the function of the cells in the mesenchyme and/or endothelial cells of the AGM remains unclear.

As demonstrated in the adult bone marrow, the maintenance and differentiation of HSCs is regulated by complex interactions with the surrounding cells; the hematopoietic microenvironment [9,30]. During ontogeny, before the establishment of the trabecular bones and bone marrow, HSCs and progenitors reside in different microenvironments; in the AGM, yolk sac, placenta and fetal liver. To study the interactions between HSCs and their microenvironment, *in vitro* co-culture systems have been developed. Stromal cell lines established from the microenvironment of the bone marrow and fetal liver were found to support HSCs and progenitors [15,35]. Recently, Oostendorp and co-workers [20] showed that stromal cell lines generated from the AGM microenvironment are able to provide high level support to HSCs and progenitors, suggesting that the AGM region provides a potent microenvironment for HSCs in the embryo. Harvey et al. [7] showed that cell-cell contact is essential *in vitro* for maintenance of HSCs on AGM-derived stromal cell lines and thus, like in the bone marrow microenvironment a role for adhesion molecules and receptor-ligand interactions is likely [8,34,36]. However, our current understanding of other molecules involved in the interactions of HSCs and progenitors within this microenvironment is poorly understood.

Since *Runx1* is expressed in the mesenchyme and endothelial cells of the AGM microenvironment [16] we set out to determine whether Runx1 affects the hematopoietic supportive capacities of AGM stromal cells. We generated stromal cell lines from AGM regions wild type or deficient for *Runx1*. We show here that the level of Runx1 within the stromal microenvironment of the AGM region affects the capacities of these cells in their support of hematopoietic progenitors and HSCs *in vitro*.

Materials and methods

Isolation of the parental and cloned AGM-derived stromal cell lines

AGM regions were dissected from *Runx1* wild type or deficient E11 embryos [19] (Figure 1A). The dissected AGMs were explant-cultured for 4-5 days at the air-medium interface on 0.1% gelatin coated 6-well plates in LTC-SM medium (50% M5300, StemCell Technologies; 15% heat-inactivated FCS, Gibco; 35% α-MEM, Gibco; penicillin and streptomycin, Gibco; 10 μM β-mercaptoethanol, Merck) at 33 °C, 5% CO_2. Cells were harvested after trypsin-EDTA exposure and were seeded on a new pre-coated dish supplemented with 10-20% (0.2 μm-filtered) supernatant from the previous passage each week until the cell numbers increased consistently. The parental *Runx1* wild type and deficient stromal cell lines were harvested by trypsin-EDTA exposure and single cells were obtained. Using the FACS-Vantage cell sorter, the cell lines were cloned by seeding 1 cell per well in pre-coated 96-well plates. After 1-2 weeks clones were harvested and expanded.

Previously described stromal cell lines used; UG26-1B6 wild type stromal cell line derived from E11 urogenital ridges subregion and the FBMD-1 adult bone marrow cell line. Both were cultured as described [1,21].

DNA PCR analysis of Runx1

Runx1 wild type and deficient stromal cell lines were genotyped by DNA-PCR analysis. PCR primer sequences are: mAML-1E4 5'-GCCATCACAGTGACCAGAGTGC-3', mAML-1I3 5'-CTGTACCAATGAGAAACAGTAGTAGC-3', TKPA up 5'- GGCAGGCCCTGCCATAGC-3' (ratio = 1:2:1). PCR reactions were performed at 1 cycle of 92°C for 5 minutes, 30-32 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute and elongated at 72°C for 10 minutes. The wild type allele (400 bp) and targeted (KO) allele (600 bp) were separated by gel electrophoresis on a 1.5% agarose/1xTBE with ethidium bromide gel.

RNA isolation, cDNA synthesis and RT-PCR analysis

Total RNA was isolated from cell lines with TRIZOL (Gibco/Life Technologies) according to manufacturer's instructions and DNAse treated with RQ1 Rnase free DNAse (Promega). For cDNA synthesis 1-5 μg of total RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen/Life Technologies) according to manufacturer's instructions. Primer sequences used to detect *Runx1* mRNA; Forw-1700 5'- AGCATGGTGGAGGTACTAGC –3' and Rev-D 5'- CCGACAAACCTGAG-

GTCGTTG –3'. PCR reactions were performed in 50 µl with 1U Amplitaq (PerkinElmer) and provided buffer, 100 ng of each primer, 1 mM dNTPs and 1-2 µl of cDNA. PCR reactions were performed at 1 cycle of 92°C for 5 minutes, 35-40 cycles of 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute and elongated at 72°C for 10 minutes. PCR products (200 bp) were run of 1.5% agarose/1xTBE gel with ethidium bromide, scanned on a Typhoon Scanner (Molecular Dynamics) and analysed with ImageQuant software.

Design and cloning of shRNA directed against murine *Runx1* and transfection into UG26-1B6 cells

A pair of 64-nucleotide oligonucleotides encoding a 19-nucleotide Runx1 shRNA (5'- GATCCCCACTACTCGGCAGAACTGAGTTCAAGAGACTCAGTTCTGCCGAGTAGTTTTTTGGAAA –3' and 5'- AGCTTTTCCAAAAAACTACTCGGCAGAACTGAGTCTCTTGAA CTCAGTTCTGCCGAGTAGTGGG –3') were designed, which contained extra *BglII* and *HindIII* restriction sites to facilitate cloning. The position of the core 19-nucleotide sequence (underlined) targeted nucleotides 1910 to 1928 of the murine *Runx1*. Blast nucleotide search was performed using the National Center for Biotechnology Information (NCBI) to ensure that the shRNA construct was targeting only murine *Runx1*. The 64-nucleotide oligonucleotides were annealed and cloned into the *BglII* and *HindIII* sites of the pSUPER.retro.neo+gfp vector (OligoEngine, Seattle) and the resulting vector (pSUPER.retro.neo+gfp-Runx1) was subsequently sequenced to confirm identity.

UG26-1B6 cells were grown as described before [21] and stably transfected with the pSUPER.retro.neo+gfp-Runx1 or the pSUPER.retro.neo+gfp-empty vector control using Fugene 6 Transfection Reagent (Roche) according to manufacturers protocol. Both vectors were linearized using the *ScaI* restriction enzym to allow incorporation into the genomic DNA of the UG26-1B6 cells. The transfected UG26-1B6 cell line was harvested by trypsin-EDTA exposure and single cells were obtained. Using the FACS-Vantage cell sorter, the transfected cells were cloned by seeding 1 GFP^+ cell per well in pre-coated 96-well plates. After 1-2 weeks clones were harvested and expanded and analysed for *Runx1* expression as described above.

Long-term co-culture of unfractionated bone marrow cells

Femurs and tibias from 3 C57BL/6J mice were collected and crushed by mortar and pestle. Single cells were collected in medium containing PBS (Gibco Inc.), 10% heat-inactivated filtered FCS (GibcoBRL) and Pen/Strep (GibcoBRL) (PBS/FCS/PS) and washed twice. The flasks containing the confluent irradiated layer of the *Runx1* deficient and UG26-1B6 control cell lines were seeded with $1x10^6$ bone marrow cells and the co-cultures were maintained for 2-6 weeks at 33°C, 5% CO_2 in IMDM supplemented with 10% heat-inactivated filtered FCS (GibcoBRL), 10% filtered horse serum (GibcoBRL), 10 µm β-mercaptoethanol (Sigma), 10^{-6}M hydrocortisone (Sigma) and Pen/Strep (GibcoBRL). Each week, half of the medium was removed and non-adherent cells were recovered. These cells were added back to the cultures, along with fresh medium. After 2, 4 and 6 weeks the production of hematopoietic

progenitors was tested by CFU-C-method (1) (described below). Quantitations of the more immature hematopoietic progenitors were performed after 6 weeks of co-culture using the CAFC method.

Enrichment of hematopoietic progenitors and HSCs from bone marrow

Femurs and tibias from 3 to 5 Bl1b transgenic [13] (C57BL10 x CBA)F_1 or *Runx1* +/- (C57BL6/J) mice were collected and crushed by mortar and pestle. Single cells were collected in PBS/FCS/PS medium. The discontinuous ficoll gradient from E. Schneider [29] was adapted for murine bone marrow cells and performed using a 30% w/w Ficoll-400 solution (31.5 gram Ficoll-400 (Pharmacia) dissolved in 100 ml 0.1 M sodium phosphate solution pH=7.4). This 30% w/w solution was diluted into 19% w/w and 22% w/w with the sodium phosphate buffer. The discontinuous gradient was layered as follows: 3.5 ml 22% w/w Ficoll-400, 2.5 ml 19% w/w Ficoll-400, 1.5 ml containing 2-3x10^8 cells in PBS/FCS/PS in Beckman Pollyallomer (14x89 mm) tubes. Ultracentrifugation in a precooled Beckman Optima^{TM}L using a swing-out SW40Ti rotor with buckets at 13.800 rpm, 35 minutes, slow acceleration and no break at 4^{o}C was performed. Low-density bone marrow cells layered between 1.069 g/ml (19% w/w) and 1.075 g/ml (22% w/w) were collected. Cells were washed twice in PBS/FCS/PS before further use.

The low-density bone marrow cells were incubated with phycoerythrin-conjugated anti-CD31 (PECAM1) (BD Pharmingen), isothyocyanate-conjugated anti-Ly-6C (ER-MP20) (BD Pharmingen) and allophycocyanin-conjugated anti-c-kit (CD117) (BD Pharmingen) antibodies for 30 minutes on ice. CD31^{med} Ly-6C$^-$ c-kit^{high} cells were sorted on the FACSAria (Becton Dickinson). Dead cells were eliminated by negatively selecting for Hoechst 33258 (Molecular Probes) labelled cells. The sorted cells were then co-cultured on top of a confluent irradiated layer of the *Runx1* deficient and UG26-1B6 control cell lines (2000 cells per well in a 6-well plate) using LTC-SM medium. After 1 week of co-culture cells were used for CFU-C assay, flow cytometric analysis and long-term transplantation.

FACS analysis

Cells were collected from the co-cultures seeded with 2000 enriched bone marrow cells and washed in PBS/FCS/PS. Single cell suspensions were labelled with several combinations of antibodies for 30 minutes on ice. CD31 (PECAM1), Mac1 (CD11b), GR-1 (Ly-6G) and CD45 antibodies were phycoerythrin-conjugated, Ly-6C (ER-MP20), Dx-5, B220 (CD45R) and Sca-1 were isothyocyanate-conjugated and c-kit (CD117) was allophycocyanin-conjugated. Dead cells were excluded after 7AAD (Molecular Probes) staining and FACS analysis was performed on FACScan (Becton Dickinson) and analysed with Cell Quest software. All antibodies used are from BD Biosciences.

Cobblestone-Area-Forming-Cell (CAFC) assay

Confluent stromal layers of FBMD-1 cells in flat-bottom 96-well plates (Falcon) were overlaid with cells harvested after 6 weeks of co-culture in a limiting dilution set up [25,26]. Briefly, 12 successive two-fold dilutions were used for each sample with 15 wells per dilution. The percentage of wells with at least one phase-dark hematopoietic cobblestone area of at least five cells beneath the stromal layer was determined at day 10, 21, 28 and 35 after plating. Freshly isolated bone marrow (control input sample)

was initiated in the cultures at $1x10^5$ cells per well plated in the first dilution, while after the co-cultures $1\text{-}2x10^5$ cells per well were used in the first dilution. The CAFC frequencies at the different time points were calculated using Poisson statistics.

In vitro hematopoietic progenitor colony assay (CFU-C)

Method (1) Cells were collected after co-culture, washed in PBS/FCS/PS and counted using a Sysmex Microcellcounter (CC-120). Cells were seeded in triplicates at concentrations varying between $2x10^4$ and $3x10^6$ cells per plate in methylcellulose medium (Methocult™ M3231; Stem Cell Techn. Inc) supplemented with 2% PWM-SCCM (HemoStim™ M2100; Stem Cell Techn. Inc) and IMDM (Gibco-BRL). Cultures were incubated at 37°C in a humidified chamber under 5% CO_2. The total number of colonies was scored with an inverted microscope at day 7 of the culture.

Method (2) Cells were collected from the co-cultures seeded with 2000 enriched bone marrow cells and washed in PBS/FCS/PS. An equivalent of 50-200 co-cultured cells were then seeded in triplicates into methylcellulose medium (Methocult™ M3434; StemCell Technologies Inc.) supplemented with stem cell factor (SCF), IL-3, IL-6 and Epo. Cultures were incubated at 37°C in a humidified chamber under 5% CO_2. Colony-forming unit-granulocyte (CFU-G), colony-forming unit-macrophage (CFU-M) and colony-forming unit-granulocyte macrophage (CFU-GM) were scored with an inverted microscope at day 7 of the culture.

In vivo transplantation assay

We performed intravenous injection of genetically marked (Bl1b transgenic [13]) cells into adult (C57BL/10×CBA)F_1 recipients that were exposed to a split dose of 9 Gy of γ-irradiation (^{137}Cs source). Recipients were injected with several doses of freshly isolated enriched bone marrow cells or an equivalent of 50-2000 co-cultured cells. 2×10^5 spleen cells (recipient background) were co-injected to promote short-term survival. Mice were maintained on 0.16% Neomycin (Sigma) water. Blood DNA was obtained at 4 months after transplantation and analysed for donor cell genetic marker by semiquantitative PCR (*LacZ* [13]). The percentage of donor chimerism was determined from fluorescence of ethidium bromide stained gels (Imagequant) and calculated from a standard curve of DNA control dilutions (0%, 1%, 3%, 10%, 30%, 60%, and 100% donor marker). Recipients were considered repopulated only when donor chimerism was more than 10%. HSC frequencies were calculated with L-Calc software (Stem Cell Techn.).

Results

Generation of the AGM-derived stromal cell lines

To investigate the role of the Runx1 transcription factor within the AGM stromal compartment and potential Runx1 related stromal cell interactions with hematopoietic progenitors and HSCs, we isolated AGMs from mouse embryos wild type or deficient for *Runx1*. The *Runx1* defective allele contains a

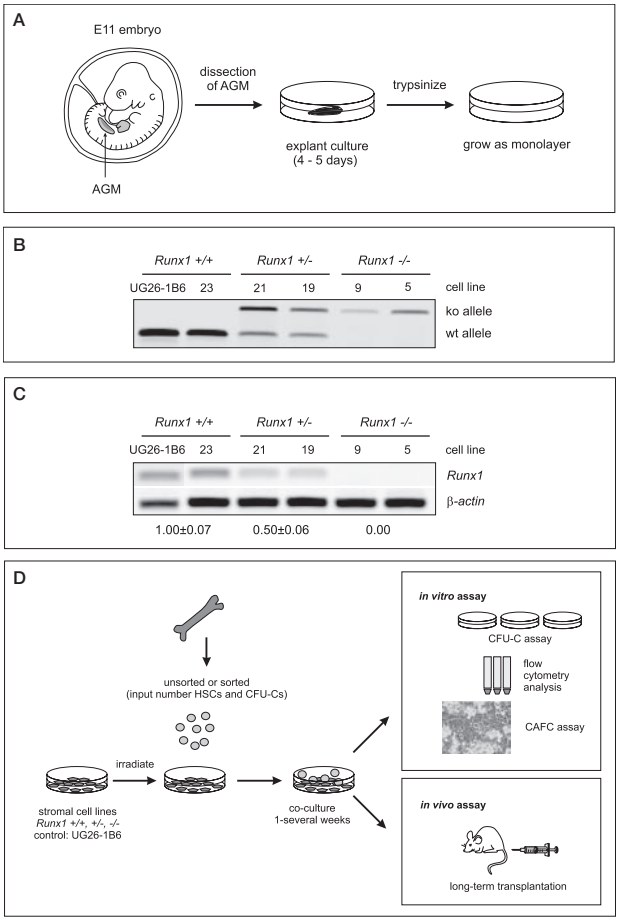


Figure 1. Generation of AGM-derived stromal cell lines wild type or deficient for *Runx1*

(A) Embryos, wild type or deficient for *Runx1*, were collected at E11 and AGM regions were dissected. Individual AGMs were explant-cultured at the air-medium interface in stromal medium. After 4-5 days non-adherent cells were collected with the supernatant and adherent cells by trypsin exposure. The harvested cells were grown as monolayers until cell numbers increased consistently. From an individual AGM one cell line was generated. **(B)** PCR genotyping of the AGM derived stromal cell lines used in this study. Each cell line was tested by DNA PCR for the presence of the wild type and targeted (= KO) *Runx1* allele. Two *Runx1* +/+ (UG26-1B6 and 23), two *Runx1* +/- (21 and 19) and two *Runx1* -/- (5 and 9) were used in this study. **(C)** RT-PCR analysis of mRNA from the stromal cell lines used in this study. *Runx1* homozygous disrupted stromal cell lines showed no detectable levels of runt-domain containing mRNA, whereas wild type or heterozygous *Runx1* stromal cell lines produced the wild type mRNA at the 100% and 50% levels respectively. The ubiquitously expressed β-*actin* mRNA was used as a normalization control for semi-quantitative analysis of *Runx1* expression levels. Relative levels are indicated as the mean (± SEM) of two individual experiments. **(D)** Protocol used for study of the hematopoietic supportive capacities of stromal cell lines. Stromal cell lines deficient for *Runx1* were seeded into 6-well plates, grown to confluency and irradiated (20 Gy). Cultures were seeded with the (enriched) HSC populations (wild type or *Runx1* +/-) and grown for 1-several weeks. Subsequently, *in vivo* and *in vitro* assays are performed to analyse HSCs and progenitors (transplantation and CFU-C or CAFC assays respectively). In addition, phenotypical analysis of the co-cultures can be performed using flow cytometry.

termination codon in the runt-domain which functions as the DNA binding domain of the protein, thus rendering it functionally inactive [19]. AGMs were isolated from E11 embryos (at a time when the HSC activity is high) and cell lines were generated (Figure 1A). DNA PCR confirmed the cell line genotype for the wild type and targeted *Runx1* allele (Figure 1B). We obtained one wild type cell line (23), two *Runx1* heterozygous disrupted cell lines (21 and 19) and two *Runx1* homozygous disrupted cell lines (5 and 9). *Runx1* expression levels were determined for each of the stromal cell lines by semi-quantitative RT-PCR using *Runx1*-specific primers that bracket the site of targeting in exon 4 [19]. As shown in Figure 1C, *Runx1* mRNA levels in heterozygous stromal cell lines (0.50±0.06) were half of the levels in wild type stromal cell lines (1.00±0.07). The homozygous *Runx1* disrupted stromal cell lines showed no

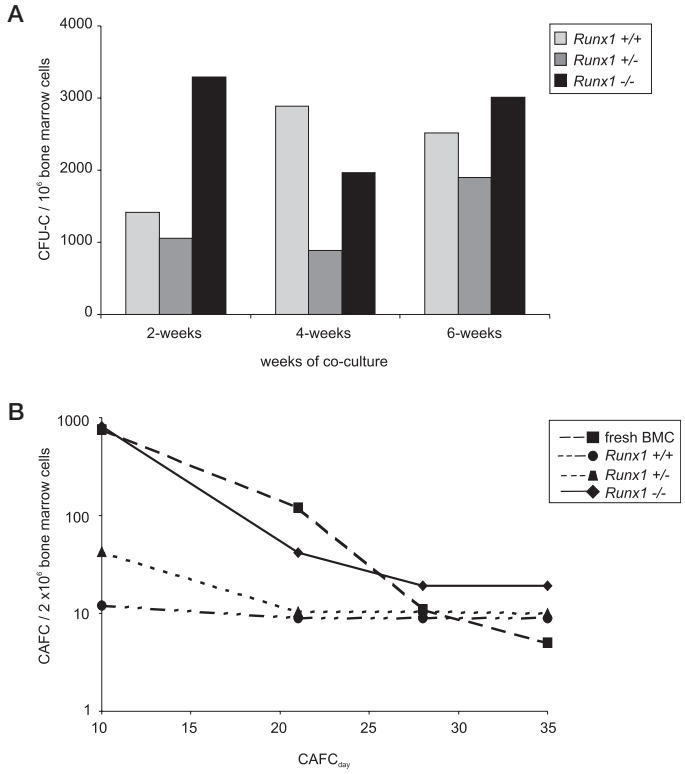


Figure 2. Long term CFU-C and CAFC supportive capacities of the *Runx1* wild type or deficient AGM-derived stromal cell lines

(A) Long term CFU-C supportive capacities of AGM stromal cell lines. The absolute number of CFU-C is shown as the number of CFU-C per 10^6 total bone marrow cells inoculated in co-culture. Every 2 weeks samples were taken to quantify the CFU-C growth during co-culture. Data is shown as the mean of 2 individual cell lines per genotype tested. n = 1 to 2 per cell line. **(B)** Long term CAFC supportive capacities of AGM stromal cell lines. CAFC assays were performed after 6 weeks of co-culture to determine mature and immature hematopoietic progenitors growth *in vitro*. Data are the arithmetic means of 2 individual cell lines per genotype tested (except the -/- genotype). n = 1 to 2 per cell line.

expression of *Runx1*. These stromal cell lines, together with the previously characterized AGM stromal cell line UG26-1B6 (*Runx1* +/+) formed the basis of the studies examining whether Runx1 affects the hematopoietic supportive capacities of the AGM microenvironment (Figure 1D).

Long-term hematopoietic supportive capacities of parental *Runx1* deficient AGM-derived stromal cell lines

To test the hematopoietic supportive capacity of AGM-derived stromal cell lines *in vitro*, unfractionated murine wild type bone marrow cells were cultured on top of *Runx1* +/+, +/- and -/- stromal cell lines for several weeks. Hematopoietic progenitor cell growth was assessed by the Colony-Forming-Unit in Culture (CFU-C) assay (method 1) (Figure 2A) after 2, 4 and 6 weeks of co-culture. In general, none of the stromal cell lines was able to maintain the input numbers of CFU-Cs (3900 CFU-C/10^6 bone marrow cells). However, *Runx1* +/+ and -/- lines showed levels of support, with CFU-C numbers increasing to almost input CFU-C levels at week 6. At all time points the *Runx1* +/- stromal cells showed the lowest level of support for bone marrow CFU-Cs (decreased 2–4 fold as compared to input). Moreover, the kinetics of support differed between the cell lines of the differing genotypes: *Runx1* -/- lines maintained high numbers of CFU-Cs at all 3 time points. Support for CFU-Cs by the *Runx1* +/+ lines reached a plateau at week 4 and *Runx1* +/- lines showed an increase in CFU-Cs only at week 6. Thus, the level of *Runx1* expression within AGM stromal cell lines appears to affect their *in vitro* supportive capacity for hematopoietic progenitors.

After 6 weeks of co-culture Cobblestone-Area-Forming-Cell (CAFC) assay was performed to assess the supportive capacity of *Runx1* +/+, +/- and -/- stromal cell lines for more mature ($CAFC_{10/21}$) and immature hematopoietic progenitors ($CAFC_{28/35}$) (Figure 2B). Freshly isolated bone marrow contained 760-120 $CAFC_{10/21}$/2x10^6 cells and 11-5 $CAFC_{28/35}$/2x10^6 cells. After co-culture with *Runx1* +/+ and +/- stromal cell lines only a low level of $CAFC_{10}$ were found (60-fold and 20-fold decrease respectively compared to input). Interestingly, the *Runx1* -/- stromal cell line maintained the same high number of hematopoietic progenitors as those found in freshly isolated bone marrow. $CAFC_{28/35}$ were also found after 6 weeks co-culture with the *Runx1* +/+ and +/- stromal cell lines and these progenitors were in equivalent numbers to those found in freshly isolated bone marrow. Surprisingly, the *Runx1* -/- stromal cell lines maintained $CAFC_{28/35}$ at 4-fold higher levels than the freshly isolated bone marrow cells. Thus, *Runx1* -/- stroma best supports the most immature hematopoietic progenitors *in vitro*. Although only one *Runx1* -/- stromal cell line was tested for long-term supportive capacities, these results suggest that the level of *Runx1* within the AGM stroma plays a dose-dependent role in hematopoietic cell support. Since unfractionated bone marrow cells comprise of a number of different cell types varying in their differentiation stage we set out to enrich bone marrow cells specifically for hematopoietic progenitors and HSCs.

Hematopoietic supportive capacities of parental *Runx1* deficient AGM-derived stromal cell lines for enriched bone marrow hematopoietic progenitors/stem cells

Using murine bone marrow cells enriched for HSCs and progenitors ("light density" CD31^{med} Ly-6C^{-} c-kit^{high} [20]) we tested the supportive capacities of *Runx1* +/+, +/- and -/- stromal cell lines in co-culture. Following 1 week of co-culture we performed phenotypic characterization using flow cytometry analysis of different hematopoietic populations. Total hematopoietic cells (CD45^{+}), immature progenitors (CD45^{+} c-kit^{+} Sca-1^{+}) and myeloid progenitors (Gr-1^{+}, Mac-1^{+} or Ly-6C^{+}) were found in all co-cultures, whereas no B-cells (B220^{+}) or natural killer cells (Dx-5^{+}) were found (data not shown). Moreover, a 1-8 fold increase (compared to input number) of CD31^{med} Ly-6C^{-} c-kit^{high} HSCs enriched population was found in all co-cultures. Thus, the *Runx1* genotype of the stromal cells does not appear to affect the percentages of hematopoietic cells supported in co-cultures or the appearance of round hematopoietic cells in the co-cultures (Figure 3; black arrows). However, the morphological appearance of the co-cultures for cobblestone areas did vary. Co-cultures of enriched bone marrow cells with the *Runx1* +/+, *Runx1* -/- and the UG26-1B6 control cell line showed abundant cobblestone areas (Figure 3; black arrowheads). Strikingly, the *Runx1* +/- stromal cell lines showed none or very few cobblestone areas.

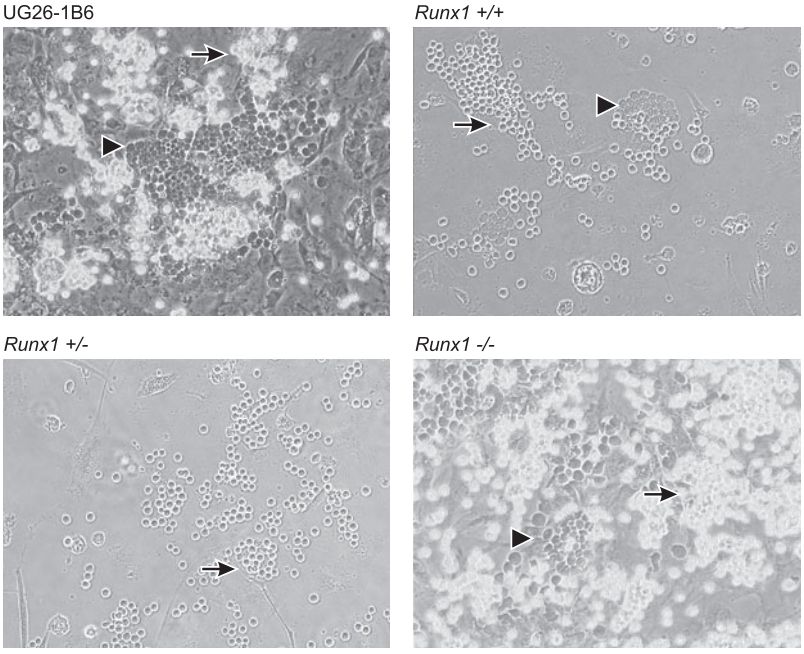


Figure 3. Co-culture morphology
After 7 days the co-culture of UG26-1B6, *Runx1* +/+ (23) and *Runx1* -/- (9) with enriched HSC bone marrow population contained cobblestone areas (black arrowheads). In contrast, co-culture with the *Runx1* +/- (21) showed little or no support of cobblestone areas. In all co-cultures hematopoietic-like colonies were present (black arrows).

We next assessed the support of functional hematopoietic progenitors after the co-culture period by the CFU-C assay (method 1). Prior to co-culture, input numbers of mature hematopoietic progenitors in the enriched "light density" CD31^{med} Ly-6C^{-} c-kit^{high} fraction of bone marrow were determined. The number of input hematopoietic progenitors (243 ± 63 per well) was increased 5.9-fold (1419 ± 465 per well) when cultured for 1 week on top of the *Runx1* +/+ control UG26-1B6. This value serves as the 100% control for hematopoietic progenitor support in the test stromal co-cultures. We found that the

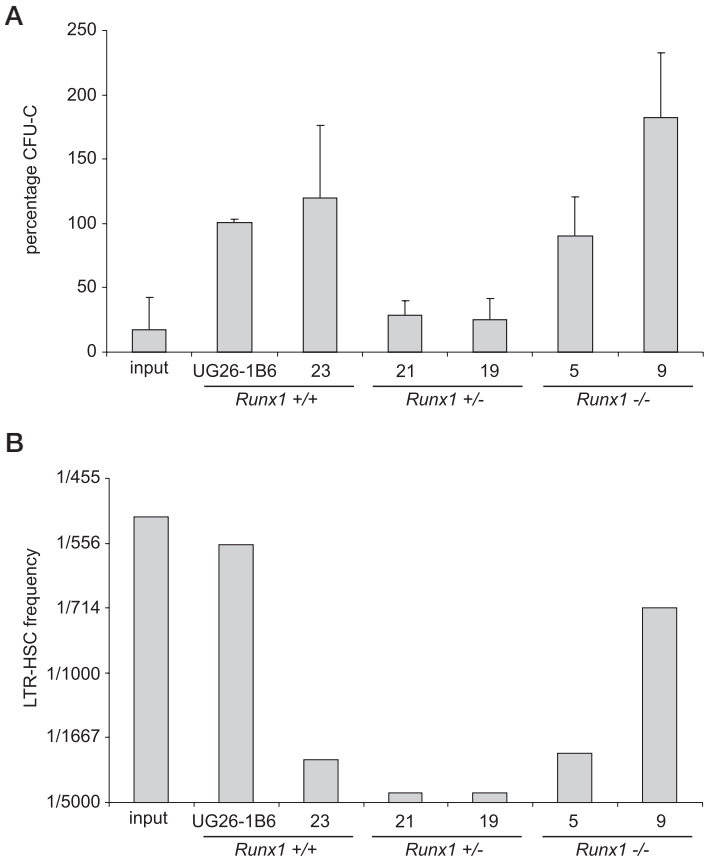


Figure 4. Hematopoietic progenitor and HSC supportive capacities of the *Runx1* wild type or deficient parental AGM-derived stromal cell lines

Analysis of co-cultures of HSC enriched bone marrow cells on *Runx1* wild type and deficient stromal cell lines. **(A)** Hematopoietic progenitor supportive capacities of stromal cell lines. On the y-axis the percentage of CFU-C is depicted. The number of CFU-Cs found after 1-week co-culture on the *Runx1* +/+ control cell line UG26-1B6 was set to be 100% and every other co-culture was expressed as a percentage of this. The graph shows the combined results of 4 experiments. **(B)** HSC supportive capacities of stromal cell lines. The bar graph shows the frequency of LTR-HSCs present in the co-cultures after 1 week. Note that the y-axis displays the frequency in a logarithmic scale. Results shown are from 4-6 experiments using Poisson-law statistics (L-Calc software) to calculate the frequency of LTR-HSCs.

Runx1 +/+ and *Runx1* -/- cell lines were able to support hematopoietic progenitors almost as well or better than UG26-1B6 (Figure 4A). In contrast, the *Runx1* +/- cell lines showed only a poor level of support for hematopoietic progenitors, maintaining only input progenitor numbers.

To determine the supportive capacity of the stromal cell lines for long-term *in viv*o repopulating HSCs, sorted bone marrow cells were co-cultured for one week, harvested and injected (at several cell doses) into irradiated adult recipient mice (Figure 4B). At 4 months post-transplantation, recipient peripheral blood DNA was examined for the donor cell contribution by a semi quantitative DNA PCR method for the donor cell marker (*LacZ* transgene [13]). The number of mice repopulated (10% or greater donor chimerism) per number of mice injected at each cell dose was determined and the frequency of HSCs within the sorted CD31^{med} Ly-6C^{-} c-kit^{high} input population was calculated. In the input population, 1 HSC in every 510 bone marrow cells was found. After co-culture with the *Runx1* +/+ UG26-1B6 control cell line, 1 out of 558 cells is an LTR-HSC. As this frequency resembles the frequency in the input population, UG26-1B6 is able to maintain HSCs as was previously reported [20]. The other *Runx1* +/+ cell line (23) showed ~4-fold decrease in support of HSCs compared to the UG26-1B6 cell line (1 out of 2100 cells). Both the *Runx1* +/- cell lines show very poor support for HSCs (1/4000) (~7-fold decrease) compared to input numbers. Strikingly, one of the *Runx1* -/- cell lines (9) supported HSCs similar to UG26-1B6 (1 out of 715 cells) while the other *Runx1* -/- cell line (5) showed only a ~4-fold decrease compared to UG26-1B6. Thus, a half dose of *Runx1* in the stroma has a negative effect on hematopoietic support while curiously, an absence of *Runx1* results in potent support for HSCs.

Hematopoietic supportive capacities of cloned *Runx1* deficient AGM-derived stromal cell lines

To verify that the supportive capacities of the *Runx1* deficient cell lines are not a consequence of cell population heterogeneity, the parental stromal cell lines, *Runx1* +/+ cell line 23, *Runx1* +/- cell line 21 and *Runx1* -/- cell line 9, were cloned. Clones wild type (23.A and 23.E), heterozygous (21. A and 21.D) and homozygous (9.B and 9.C) disrupted for *Runx1* were tested for their hematopoietic progenitor support as previously done with the parental cell lines (Figure 5; CFU-C method 2). The total number CFU-Cs of the *Runx1* +/+ enriched bone marrow population (1156 ± 317 per well) was increased during co-culture for 1 week on the *Runx1* +/+ control cell line UG26-1B6 (1703 ± 603 per well) (Figure 5A). The increase in CFU-C during the co-culture suggests that the cell line increases the production of CFU-Cs from immature progenitors and HSCs and/or can support the preservation of inoculated CFU-Cs. Again the *Runx1* +/+ and -/- cell lines provided the best support (88% to 138% compared to UG26-1B6 control co-cultures), with the *Runx1* +/- cell lines unable to efficiently sustain CFU-Cs (30% compared to UG26-1B6 control co-culture). Cloned *Runx1* +/+ and -/- stromal cell lines supported hematopoietic progenitors (Figure 5A) similar to or better than the parental cell lines, while the *Runx1* +/- clones showed decreased support. Moreover, in all co-cultures with the cloned stromal cells all kinds of progenitors were detected (CFU-G, M and GM; data not shown) and did not vary from the colonies observed after co-culture with parental lines.

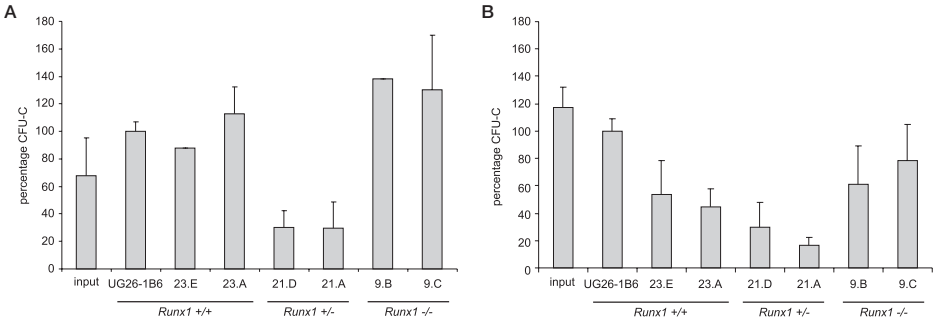


Figure 5. Hematopoietic progenitor supportive capacities of the *Runx1* wild type or deficient AGM-derived stromal cell clones

Analysis of CFU-Cs from co-cultures of **(A)** *Runx1* +/+ and **(B)** *Runx1* +/- HSC enriched bone marrow cells on *Runx1* wild type and deficient stromal clones. On the y-axis the percentage of CFU-Cs after 1 week of co-culture are depicted. The number of CFU-Cs found on the *Runx1* +/+ control cell line UG26-1B6 was set to be 100% and every other co-culture was expressed as a percentage of this. Both graphs show the results of 3-5 experiments.

Since we found that quantitative levels of *Runx1* appear to have an intrinsic affect on AGM stromal cell function, it was interesting to test whether *Runx1* +/- HSCs and progenitors in co-cultures are supported similarly to their *Runx1* +/+ counterparts. From literature it is already known that adult *Runx1* +/- mice have elevated levels of hematopoietic progenitors and only half the number of HSCs [31] in their bone marrow. *Runx1* +/- bone marrow cells were enriched for HSCs and progenitors ("light density" $CD31^{med}$ Ly-$6C^{-}$ c-kit^{high}) and were seeded on the stromal cell clones. Input numbers of CFU-Cs (414 ± 63 per well) were reduced in all the co-cultures. The average reductions were: *Runx1* +/+ ~2-fold, *Runx1* +/- ~5-fold, *Runx1* -/- ~2-fold. *Runx1* +/+ and -/- cloned stromal cell lines were found to support hematopoietic progenitors better than *Runx1* +/- cloned stromal cell lines (3-fold difference), following the same general pattern of support as for the *Runx1* +/+ enriched bone marrow cells (Figure 5B). Moreover, the cloned stromal cell lines supported all types of *Runx1* +/- progenitors (CFU-G, M and GM; data not shown). These results suggest that there are AGM stromal cell intrinsic affects (as well as adult bone marrow hematopoietic progenitor/stem cell affects) related to *Runx1* levels of expression.

Currently, we are analysing the supportive capacities of the cloned stromal cell lines for long-term repopulating HSCs. Preliminary results were obtained using *Runx1* +/- bone marrow cells as HSC source (data not shown). So far, *Runx1* +/+ and -/- cloned stromal cell lines are able to support HSCs after co-culture (HSC frequency 1/4581 and 1/2799 respectively), whereas *Runx1* +/- cloned stromal cell lines fail to support HSCs (0 recipients repopulated out of 15 transplanted). Future experiments will use *Runx1* +/+ bone marrow cells in co-cultures with cloned stromal cell lines to determine HSC supportive capacity.

RNAi mediated *Runx1* downregulation affects stromal cell hematopoietic support

Although AGM stromal cells were cloned, variation in the hematopoietic support by cells of the identical *Runx1* genotype persisted suggesting other intrinsic molecular differences. These could be related to cell lineage, as stromal cells differ in mesenchymal lineage potential and represent cells at different branch points of this lineage differentiation hierarchy. They could also be related to molecular changes occurring in culture to allow the unlimited *in vitro* cell propagation. To eliminate such

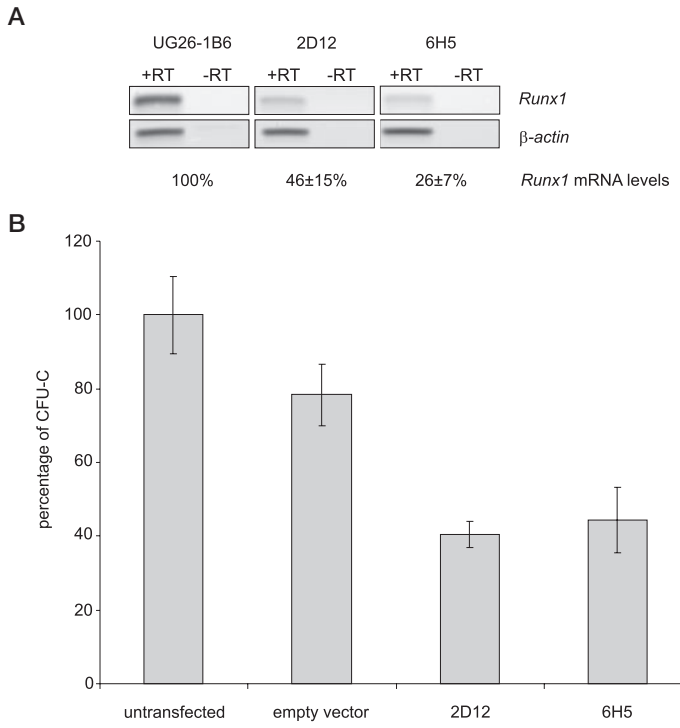


Figure 6. Hematopoietic progenitor supportive capacities of *Runx1*-RNAi down-regulated stromal cell clones

(A) RT-PCR analysis of mRNA from the *Runx1*-RNAi transfected stromal cell lines. The untransfected UG26-1B6 stromal cell line expresses normal levels (100%) of *Runx1* mRNA, whereas 2 transfected clones, 2D12 and 6H5, show reduced *Runx1* mRNA levels (46±15% and 26±7% respectively). RT-PCR was performed for the ubiquitously expressed β-*actin* mRNA as a normalization control. Relative levels are indicated as the mean (± SEM) of at least three individual experiments. RT = reverse transcriptase **(B)** Hematopoietic progenitor supportive capacities of *Runx1* down-regulated AGM stromal cell clones for HSC enriched bone marrow cells in co-culture. On the y-axis the percentage of CFU-Cs after 1 week of co-culture are depicted, with the untransfected UG26-1B6 clone serving as the 100% control. CFU-C numbers determined from the other co-cultures were expressed as a percentage of the UG26-1B6 control. The graph shows the results of multiple experiments.

heterogeneity so as to better assess the role of Runx1 in the AGM microenvironment, we transfected the well-characterized stromal clone, UG26-1B6, with *Runx1*-RNAi expression constructs.

One RNAi construct was found to downregulate *Runx1* expression in transfected UG26-1B6 cells. Clone 2D12 expressed ~50% and clone 6H5 expressed ~30% of *Runx1* mRNA levels as compared to UG26-1B6 cells or empty vector control transfected UG26-1B6 cells (Figure 6A). Co-cultures were established for each of these clones with adult bone marrow cells (enriched for HSCs and progenitors). CFU-C numbers were assessed after 1 week (Figure 6B). As expected, the untransfected UG26-1B6 and the empty vector control cell line are able to support hematopoietic progenitors after co-culture. When *Runx1* expression levels are reduced, a decrease in the support of hematopoietic progenitors is detected, with ~2.5-fold fewer CFU-Cs supported by clones 2D12 and 6H5. Thus, *Runx1* haploinsufficiency appears to result in a unfavourable microenvironment for hematopoietic progenitor growth. Whether this holds true also for HSC support is unclear. Preliminary results show that while the untransfected UG26-1B6 and the empty vector control are able to support *Runx1* +/+ HSCs (50% and 33% of recipients are repopulated respectively), the 2D12 and 6H5 clones are reduced in their ability to support long-term repopulating HSCs (19% and 13% of recipients are repopulated respectively). To further determine whether *Runx1* haploinsufficiency is incompatible with hematopoietic support, a reciprocal approach will be taken with a hematopoietic supportive *Runx1* -/- stromal clone. Future experimentation includes the generation of *Runx1* -/- stromal cell clones engineered to express *Runx1* at a single copy level (50%) to test whether hematopoietic support is decreased.

Discussion and conclusion

Runx1 is known to play an intrinsic role in the HSC compartment during embryonic development. To study the effect of the transcription factor *Runx1* on the supportive capacities of the AGM microenvironment we have generated stromal cell lines with different levels of *Runx1*. Half the dose of *Runx1* within the AGM stromal cell lines decreased their capacity to maintain hematopoietic progenitors and HSCs in culture. We confirmed this result by using RNA interference in the well-known supportive UG26-1B6 cell line, where we obtained a reduction of *Runx1* levels to 30-50% of wild type. Strikingly, the *Runx1* -/- cell lines were able to maintain the progenitor and HSCs activity at similar levels as the *Runx1* +/+ cell lines. The hematopoietic support seems independent of the genotype of the donor hematopoietic cell population. These data show for the first time that *Runx1* does not only play a role in HSCs and progenitors, but also influences the hematopoietic supportive capacity of the AGM microenvironment.

An intrinsic role of *Runx1* in the AGM stromal compartment?

Runx1 deficient embryos die between E12.5 and E13.5 with massive haemorrhages and a complete absence of HSCs in the fetal liver and AGM (no hematopoietic clusters [2]) [19,33]. Embryos haploinsufficient for *Runx1* survive but have a decreased number of HSCs in the AGM region [2,27]. In these embryos the

spatial and temporal distribution of HSCs is also disturbed. During mid-gestation *Runx1* is expressed in hematopoietic tissues [16], like vitelline and umbilical arteries [3], yolk sac, AGM region, fetal liver and the vascular labyrinth of the placenta [5,22]. More specifically, *Runx1* expressing cells in the AGM region are found in the hematopoietic clusters. Studies show that *Runx1* plays an intrinsic role in HSCs during embryonic development [19,28,33]. However, beside *Runx1* expression within the hematopoietic clusters, expression is also detected in the endothelium lining the aorta and in the underlying mesenchyme [17]. Previously, a panel of stromal cell lines [21] representing the *ex vivo* equivalent of these AGM cells was generated. We found that many of these cell lines express *Runx1* transcripts (Figure 6A and data not shown). Thus, taken together the expression of *Runx1 in vivo* in the AGM microenvironment and *ex vivo* in AGM stromal lines suggested that *Runx1* might play an important role in the stromal compartment at least during this early stage of development. Indeed, the results of our functional studies support a cell intrinsic role for *Runx1* in the AGM microenvironment. It will be interesting to determine in future experiments if this role persists at later stages.

The importance is in the level of *Runx1*

In vivo studies previously demonstrated that differential functional affects of *Runx1* dosage on hematopoiesis in the mouse embryo. To specifically investigate whether *Runx1* dosage affects the stromal compartment, we generated cell lines from wild type or *Runx1* deficient AGM tissues (Figure 1A). These cell lines were tested for their capacity to maintain/expand progenitors and HSCs in culture. Intermediate levels of *Runx1*, obtained in *Runx1* +/- cell lines or after RNAi downregulation of *Runx1* in the cell lines, show a decreased support of HSCs and progenitors. Surprisingly, when no *Runx1* is present in the stromal cell lines the supportive capacity is restored to levels detected using *Runx1* +/+ cell lines. Several explanations for this surprising discrepancy are provided:

(1) The similar support provided by the *Runx1* +/+ and -/- stromal cell lines suggest that in absence of *Runx1* other transcription factors could replace Runx1 in its function. Goyama and co-workers [6], have recently demonstrated the rescue of the *Runx1* -/- hematopoietic defect in the AGM region by *Runx2* and *Runx3* (family members of Runx1), suggesting functional redundancy between these transcription factors in early hematopoietic development. *Runx2* is highly expressed in osteoblast cells, which compose the niche where HSCs reside in the adult bone marrow. Osteoblast progenitors are present in the AGM region [12]. Thus, Runx2 may replace Runx1 within the AGM microenvironment by activating the same downstream targets. Future experiments will compare the expression profile of *Runx1* +/+ and -/- AGM stromal cell lines.

(2) We observed a decrease in supportive capacity for HSCs and progenitors using our *Runx1* +/- cell lines compared to *Runx1* +/+ and -/- cell lines. Runx1 can activate or repress target gene expression by recruitment of co-activators or repressors. When the level of *Runx1* is normal in the AGM microenvironment, activation and/or repression of target genes are compatible with efficient support of HSCs and progenitors. Half the dose of *Runx1* might change the balance between activation and/or repression resulting in a lower support. Because *Runx1* is still present in the *Runx1* +/- AGM microenvironment, no other transcription factors replace it. The existence of Runx1 in a larger complex

of factors has recently been suggested and it will be interesting to determine if such large complexes also exist in AGM stromal cells.

(3) Analysis of *Runx1* +/- embryos has shown that HSCs disappear prematurely at E11 from the AGM region [2]. Within the *Runx1* +/- AGM microenvironment, the activation and/or repression of target genes could result in a disturbed communication between stromal cells and HSCs. Thereby the AGM microenvironment is no longer able to maintain HSCs, which will die or will prematurely migrate out of the AGM. While all our *Runx1* deficient stromal cell lines were derived from E11 AGMs, it would be interesting to generate stromal cell lines from E10 AGMs. According to Cai and co-workers [2], the HSC activity in E10 AGM regions from *Runx1* +/+ and +/- embryos is similar. Thus we might expect a similar hematopoietic support from *Runx1* +/+ and +/- E10 stromal cell lines.

Does *Runx1* play a different role in different hematopoietic sites?

Haploinsuficiency for *Runx1* in E11 yolk sac [2] and placenta [27] show higher numbers of HSCs as compared to wild type. This is in contrast to the E11 AGM region, where haploinsuficiency leads to reduction of HSCs (50%) as compared to wild type [2,27]. This opposing effect of *Runx1* haploinsufficiency in the different embryonic sites suggests that the full dose of *Runx1* suppresses the appearance of HSCs in the placenta and yolk sac, but promotes it in the AGM. When the bone marrow of *Runx1* +/- mice was analysed elevated levels of hematopoietic progenitors, but lower levels of HSCs were detected [31]. Within all these tissues, the effects observed by the different levels of *Runx1* were interpreted to be intrinsic to hematopoietic cells. Since we have shown here that the level of *Runx1* also affects the function of the AGM microenvironment, an effect in the microenvironment of the other hematopoietic tissues can be expected. Derivation and testing of *Runx1* deficient cell lines from the yolk sac, placenta and bone marrow is important to address this question. Alternatively, an *in vivo* approach in the bone marrow can be taken. For this we set up transplantation experiments where wild type bone marrow cells were injected into *Runx1* +/+ and +/- recipients. Preliminary results show that *Runx1*+/- bone marrow microenvironment provides an increased level of support for short-term HSCs. In contrast, support for long-term HSC activity seems to be decreased in *Runx1* +/- bone marrow microenvironments (data not shown).

Since the hematopoietic supportive bone marrow niche consists of osteoblasts, in yet other experiments we analysed the mesenchymal differentiation potential of *Runx1* +/- bone marrow cells. Our preliminary results indicate a decrease (~3-fold) in the osteogenic differentiation potential (data not shown) of *Runx1* +/- bone marrow cells as compared to wild type cells. Interestingly, AGM-derived stromal cell lines have the potential to differentiate to most of the mesenchymal lineages [4]. Moreover, Mendes and co-workers showed that mesenchymal progenitors are present at all the major hematopoietic sites during mouse embryonic development, including the AGM [12]. They showed further that *Runx1* -/- AGMs possess the same number and types of mesenchymal progenitors as wild type AGMs. Unfortunately, analysis of *Runx1* +/- AGM regions (E11) was not performed. Future experiments should examine the cell intrinsic affects of *Runx1* haploinsufficiency on osteogenic differentiation of AGM cells.

Downstream targets of *Runx1* within the microenvironment

The Runx1 transcription factor regulates the expression of its target genes by dimerizing with its partner CBFβ forming a complex, which will bind to promoters and/or enhancers. Target gene expression can be up- or downregulated depending on the presence of co-activators or repressors. Some hematopoietic growth factors and receptors, e.g. interleukin-3 (IL-3) [10], GM-CSF [32], M-CSF receptor [37,38] are known target genes of Runx1 in the adult. IL-3 has recently been shown to be responsible for parts of the HSC defect observed in *Runx1* +/- embryos. *Runx1* +/- and *IL-3* +/- AGM regions show a similar HSCs defect [27]. When *Runx1* was absent or reduced, *IL-3* expression was lost or decreased accordingly, indicating that *IL-3* is a target gene of Runx1 in the embryo. Although this paper clearly shows a connection between IL-3 and Runx1 within the embryo, the expression pattern appears not to be consist with a role in the AGM stroma per se, since IL-3 producing cells are circulating [27]. To date, most commonly known targets of Runx1 reside in the adult hematopoietic lymphoid cells [23]. Target genes within the endothelium and mesenchymal cells are unknown. Given the paucity of data on Runx1 target genes in the embryo, our panel of *Runx1* wild type and deficient stromal cell lines provide a powerful tool to screen for target genes, which influence the growth of HSCs and progenitors during early development.

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Chapter 6

General discussion and future prospects

Throughout the development of the hematopoietic system, HSCs and hematopoietic progenitors (HPCs) become associated with different microenvironments in several anatomical sites. The first definitive adult-repopulating HSCs emerge at E10.5 within the AGM region of the embryo [14]. From E11 onwards they are found within the yolk sac, fetal liver, placenta and the circulation [7,11,16,30]. Around birth HSCs and HPCs are thought to migrate to the bone marrow where they reside throughout adulthood. The composition of the bone marrow stromal microenvironment is a mixture of mesenchymal cells and hematopoietic cells at various stages of differentiation. These cells produce growth factors and extracellular matrix components. It has been suggested that the embryonic tissues might provide a hematopoietic supportive microenvironment with similar mesenchymal cells, although only for a short window of time. Evidence supporting such temporal mesenchymal microenvironments comes mainly from the study of stromal cell lines generated from these embryonic tissues. Stromal cell lines from fetal liver, yolk sac and AGM support hematopoietic cells *in vitro* [15,25,40] and are thought to provide signals that influence emergence, survival, proliferation, differentiation and migration of HSCs and HPCs. Currently, most studies indicating that HSCs and HPCs are indeed regulated in their proliferation and differentiation by cells of the microenvironment *in vivo* comes from analysis of the adult bone marrow [6,18,22,34], particularly through the analysis of gene targeted mice. Overall, the communication between HSCs and HPCs with their microenvironment appears to be a highly regulated process. The studies presented in this thesis aimed to gain more insights into the role of the embryonic microenvironment and the regulation of hematopoiesis. Particularly, we focussed on the growth factors of the Fibroblast Growth Factor (FGF) family and Interleukin-1 (IL-1) and on the Runx1 transcription factor.

FGFs and the microenvironment

FGFs are a large family of growth factors implicated in different developmental processes, angiogenesis [8], wound healing [32] and hematopoiesis [3,5]. Upon binding of the FGFs to their receptors they transmit signals through several intracellular transduction pathways, influencing cellular behaviour. Several studies already revealed that FGFs play a role in the adult hematopoietic system [1,3,24,39]. In our study we used a one-step long-term bone marrow culture (LTBMC) system to show that FGF effects are mouse strain-specific. Exogenously added FGF1 and 2 to the LTBMCs of several mouse strains positively influenced the output number of LSK cells. However, FGF9 positively affected the number of LSK cells in LTBMCs of only the 129Sv mouse strain. These differences in the cultures of various mouse strains were complemented by the fact that 129Sv and C57BL6 LSK cells differentially express *Fgfr* transcripts. It would be interesting to determine whether the bone marrow stromal compartment of the mouse strains also differentially expresses these and other components of the FGF signalling pathway. By establishing a bone marrow derived stromal layer *in vitro* from these mouse strains, which are depleted for hematopoietic activity, we could examine expression patterns exclusively within the stromal compartment. Since in our LTBMCs the stromal cells were morphologically affected by addition of the FGFs, these established bone marrow stromal layers could be used in a co-culture system addressing the supportive capacities with or without exogenously added FGFs in a more controlled

manner. Using the co-culture system instead of the one-step LTBMCs, the observed effect of FGFs on the development of the stromal layers can be circumvented giving each culture an identical start.

At the onset of my thesis work, it was largely unknown whether FGFs play a role in the hematopoiesis during embryonic development. A recent study by Oostendorp and co-workers [26] revealed that AGM-derived stromal cell lines that are able to support HSCs and HPCs efficiently *in vitro*, express elevated levels of *Fgf7* and *Fgf9* transcripts. In our study we show for the first time that the AGM-derived stromal cell line UG26-1B6, which is a good supporter for HSCs and HPCs, expresses *Fgfrs*. Hence, the FGF signalling pathway could be active and function in these AGM mesenchymal cell types. Using our *in vitro* co-culture system we showed that upon addition of FGFs, the supportive capacity of the UG26-1B6 cells is influenced. Furthermore, FGF addition modulated the transcript expression of some regulators of mobilization as well as *Fgfrs*. This supports the idea that the FGF signalling pathway could influence hematopoiesis in the AGM region through its effects on the stromal cell compartment. We could perform explant cultures from E11 AGMs with or without exogenously added FGFs to address their effects on HSCs and HPCs. To expand our view on FGF signalling and hematopoiesis during embryonic development, future studies should also include other hematopoietic tissues, e.g. yolk sac and fetal liver.

Within the adult blood system, others and we showed the presence of FGFRs on the surface of immature and mature hematopoietic cells [1,3]. Currently, it is thought that FGFs affect these cells directly. An indirect influence via the stroma surrounding these cells was also suggested. As HSCs and HPCs emerge during embryonic development in the AGM, our studies showed that FGF signalling could influence these cells indirectly via the AGM stromal cells. Expression analysis of embryonic-derived HSCs for components of the FGF signalling pathway, especially the FGFRs, are necessary to reveal whether a direct effect of FGFs is also possible, as it was suggested in the adult system.

IL-1 influences the mid-gestation microenvironment and hematopoiesis

Previous studies in our lab showed that TAB2 (*Map3k7ip2*) is upregulated between E10 and E11 in the mouse aorta in/near the endothelium [28]. The temporal expression pattern of TAB2 in the aorta region correlates with that of the emerging HSCs [28]. IL-1 is a cytokine that has been reported to regulate HSCs in adult mice and TAB2 was implicated as a mediator of IL-1 signalling [10,36]. Thus, embryonic expression of TAB2 raised the question whether IL-1 could also be a regulator of HSCs and HPCs during early development.

We showed that IL-1 and its signalling components are present and functional in the AGM region and fetal liver (E11 and E12). Within the AGM region endothelial, mesenchymal and hematopoietic cells express IL-1R. Further analysis showed that IL-1 is able to affect the number of HPCs and HSCs in the AGM region after explant culture and HPCs are increased in a dose dependent manner. Low levels of IL-1 increased the number of HSCs (and CFU-GEMM) and high concentrations severely decreased their activity. In contrast, analysis of fetal liver (E11) explants cultured with exogenously added IL-1 showed no changes in HSC activity, but did stimulate an increase of the HPC activity. This

striking difference in IL-1 mediated stimulation of AGM and fetal liver HSCs suggests that IL-1 action is dependent on the hematopoietic site during development. How IL-1 may affect HSCs and HPCs present in the yolk sac and placenta, two other important hematopoietic sites during development remains to be investigated.

Closer examination of the AGM region showed that the majority of IL-1R expressing cells are mesenchymal cells. AGM- and fetal liver-derived (E11) stromal cell lines, which are of mesenchymal lineage, expressed the IL-1R as well [26]. These stromal cell lines were previously shown to support HSCs *in vitro* [25]. In fact, a high level of IL-1R expression was correlated with the long-term support of enriched HSCs. Analysis of one of these stromal cell lines, the AGM-derived UG26-1B6, showed that, upon IL-1 addition, the expression of molecules involved in the mobilization of HSCs are changed, e.g. prolonged IL-1β stimulation resulted in downregulation of *Cxcl12* isoforms. This suggested that IL-1 could act indirectly (through the stroma) on HSCs and HPCs in the AGM region. Analysis of IL-1R expression in the fetal liver (E11) showed that 60-90% of IL-1R expressing cells are not expressing hematopoietic markers. Furthermore, immunostaining revealed IL-1R expression in fetal liver is localized to the mesenchymal cell regions. Since fetal liver-derived stromal cell lines also express the IL-1R they should be able to respond to IL-1 stimulation. It would be interesting to determine whether a fetal liver-derived stromal cell line able to highly support HSCs *in vitro*, like EL08-1D2, is modulated by IL-1 in its expression of molecules involved in cross-talk between hematopoietic cells and stroma (as was UG26-1B6).

It is thought that at E11 the fetal liver is first colonized by HSCs from the AGM region and later on (E12-13) by yolk sac and placenta HSCs [7,11]. The fetal liver itself does not generate HSCs at either E11 or E12 [11] suggesting that (at these time points) the total number of HSCs present in the fetal liver is the sum of immigrant AGM-, yolk sac and placenta-derived HSCs. Thus the fetal liver microenvironment supports the homing and maintenance of AGM-, yolk sac and placenta-derived HSCs. In our studies IL-1 was added to E11 fetal livers explants, hereby only affecting HPCs and not the HSC numbers. This suggests that the maintenance of HSCs at E11 in the fetal liver is not influenced by IL-1. Later in development (E13 onwards) the number of HSCs in the fetal liver increases massively [11]. This is thought not to be the exclusive result of the homing of HSCs from other hematopoietic sites, but is also related to a shift in the function of the microenvironment from homing and maintenance to expansion of HSCs. Thus, it would be interesting to examine what the effect of IL-1 is on E13 (and onwards) fetal liver explants to determine whether IL-1 can also affect the expansion of HSCs, as we have shown in the AGM region (E11).

The maintenance of HSCs and HPCs in different hematopoietic tissues can also be the result of preventing them to undergo apoptosis [4,21,29]. Explants of E11 fetal liver cells with IL-1 showed an increase in the number of HPCs, which could implicate a cell survival function of IL-1 in this tissue. This was confirmed by the modulated expression of anti-apoptotic genes (Bcl-x) and decreased apoptosis in HPCs (AnnexinV stainings) in fetal liver explants stimulated with IL-1. It would be interesting to examine whether this increased cell survival could be the result of changes in the stromal cells of the

fetal liver. Therefore we could examine the expression of anti-apoptotic genes in a fetal liver-derived stromal cell line after stimulation with IL-1.

Runx1 and its role in the embryonic microenvironment

Runx1 +/- embryos showed a decreased number of HSCs in the AGM region (E11), whereas in *Runx1* -/- embryos no HSCs were found [2]. Moreover, the absence of hematopoietic clusters in *Runx1* -/- AGMs demonstrated that Runx1 is necessary for the emergence of HSCs. However, it is unknown whether Runx1 plays a role in the maintenance and/or proliferation of HSCs. Besides acting cell autonomously, the Runx1 expression pattern in the mesenchyme of deficient AGM regions suggested that the stromal compartment of *Runx1* +/- and -/- AGMs could be changed in its support for HSCs.

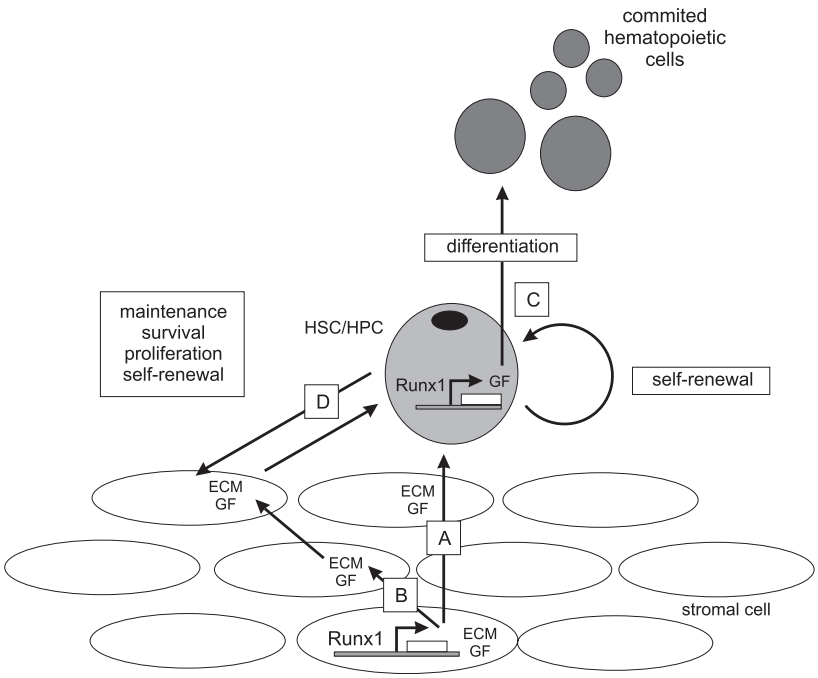

Figure 1. The role(s) of Runx1 in the embryonic microenvironment
The model displays the possible roles of Runx1 in the regulation of embryonic HSCs. Runx1 expressed in stromal cells could influence the HSCs and/or HPCs by modulating the expression of extracellular matrix (ECM) components and/or growth factors (GF) by the stromal cells that would act directly on HSCs **(A)** or on other stromal cells **(B)** that, in turn would produce other GF and/or ECM that would influence HSCs. Runx1 is intrinsically expressed in the HSCs and HPCs and could therefore modulate the expression of GF acting directly on themselves **(C)** or on the stroma **(D)**. This way Runx1 could be involved in the decisions dictating differentiation and self-renewal. All proposed pathways could affect the maintenance, survival, proliferation and self-renewal of HSCs and/or HPCs.

In our study in Chapter 5 we show for the first time that Runx1 can play a role in the AGM microenvironment. Using an *in vitro* co-culture system to analyse the effects of *Runx1* deficiency on the supportive capacities of AGM-derived stromal cell lines, we obtained the following results. First, *Runx1* is normally expressed in stromal cell lines generated from the AGM. Second, *Runx1* +/+ and -/- stromal cell lines provided support for HSCs and HPCs, whereas the *Runx1* +/- stromal cells lines provided only poor support for these cells. The reduction in the supportive capacities of *Runx1* +/- stromal cell lines together with the fact that *Runx1* +/- (E11) embryos show a reduction in the number of HSCs in the AGM explants [2] suggest that Runx1 plays a role in the maintenance and/or proliferation of HSCs upon their production in the AGM. The absence of HSCs observed in the *Runx1* -/- (E11) embryo is a hematopoietic cell intrinsic defect and is probably not a consequence of a lack of support from the microenvironment, because it is known that *in vivo* Runx1 is required for HSC emergence at E10. However, the role of Runx1 in the maintenance of HSCs via the microenvironment cannot be tested in conventional *Runx1* -/- embryos. To address this question *in vivo* in the future conditional knockout strategies in which Runx1 is targeted in the stromal compartment are necessary.

The direct precursors of HSCs (pre-HSCs) in the AGM region are thought to be either in the endothelium (haemogenic endothelial cells) and/or the mesenchyme (haemangioblast) underlying the hematopoietic clusters. In fact, it has been suggested that mesenchymal precursors could colonize the endothelium lining the aortic wall giving rise to haemogenic endothelial cells [37]. These haemogenic endothelial cells could give rise to HSCs that organize in hematopoietic clusters attached to the endothelium. It was previously reported that Runx1 expression is found in endothelial and mesenchymal cells at E10 in the AGM region [19]. Moreover, at E11 these endothelial and mesenchymal cells showed HSC activity [20]. In the Runx1 knockout models endothelial and mesenchymal cells are present in the AGM region, both at E10 and E11, but there are no hematopoietic clusters [19,23,38]. It was suggested that Runx1 could be part in the fate control of HSC emergence. Since in our study Runx1 deficiency affects the function of the stromal compartment, we can hypothesize that Runx1 influences the emergence of HSCs from haemogenic endothelium. In agreement with this hypothesis the number of endothelial HSCs (expressing VE-cadherin and CD31 but not CD45 and providing adult long-term repopulation) are dependent on the dose of Runx1 [20]. Overall, North and co-workers [20] suggested that Runx1 expression is essential for the differentiation of mesenchymal cells into haemogenic endothelium, of which the latter will transit to HSCs in the clusters. Runx1 expressing cells of the mesenchyme at E10 could influence this transition process by expression and/or repression of important growth factors. At this moment the origin, localisation and the precise phenotype of the pre-HSC are unknown. This makes it impossible to see if pre-HSCs are affected in the Runx1 deficient embryos. When in the future markers will become available it would be interesting to see whether the lack of Runx1 expression affects the pre-HSCs population in their transition to become HSCs. Within the *Runx1* deficient embryos the number of pre-HSCs might be similar as in wild type, but the transition towards HSCs in the clusters could be disrupted or the cells could not be maintained.

Analysis of *Runx1* deficient embryos previously showed that there are striking differences between HSCs present in different anatomical sites [2,33,35]. Within the yolk sac and placenta haploinsufficiency

led to increased numbers of HSCs, whereas in the AGM region and adult bone marrow only half the numbers of HSCs compared to wild type were present. It is possible that these differences reflect different forms of the Runx1 protein. Analysis of the Runx1 protein showed that several isoforms exist due to alternative mRNA splicing and there is also different promoter usage [12]. Furthermore, Runx1 exists in complexes with other factors to affect transcription. Runx1 is able to bind co-activators as well as repressors depending on their presence in specific cell types. Both the different isoforms and the binding of co-factors play a role in the activation and/or repression of target genes, ultimately leading to differences in cellular responses. This is most likely dependent on the cell type or tissue, which could express both different Runx1 isoforms and co-factors. This could also explain more subtle differences in HSC content of the hematopoietic tissues during ontogeny. Analysis of Runx1 isoforms and to which co-factors they bind in our wild type and *Runx1* deficient AGM stromal cell lines should provide us with a more molecular insight to why this tissue differentially supports HSCs and HPCs upon changed Runx1 doses. It would be further interesting to compare the supportive capacity of cell lines derived from placenta or yolk sac with our AGM-derived cell lines from *Runx1* +/- embryos. From our *in vivo* studies, it may be expected that *Runx1* +/- yolk sac and placental stromal cells are more supportive of HSCs. From these lines it can be determined if different Runx1 isoforms are present and to which co-factors Runx1 binds as compared to the AGM-derived cell lines.

Runx1 is part of a complex regulatory network that interfaces with other signalling pathways [13] to control hematopoiesis in the embryo. The function of Runx1 specifically in the stroma of the AGM region can therefore be very complex. Recently it was suggested that Runx1 could cooperate with the BMP4 signalling pathway [31]. Control of the BMP dose is essential for normal development of mesodermal lineages and hematopoiesis [9]. Regulators of the BMP4 pathway, SMAD1 and SMAD6, were shown to regulate the activity of Runx1 (SMAD1 activates and SMAD6 inhibits). Moreover, within the AGM region (E10.5) Runx1, BMP4 and SMAD6 transcripts are all detected along the ventral aspect of the dorsal aorta (mesenchymal and endothelial areas) from where the first HSCs emerge [31]. Cooperation between BMP4 and Runx1 in the supportive capacity of the mesenchymal cells comprising the AGM microenvironment for HSCs and HPCs has not been studied before. Therefore it would be interesting to determine whether BMP4 influences the expression of Runx1 and the support of HSCs and HPCs by our stromal cell lines (wild type or deficient for Runx1).

The cross-talk between hematopoietic cells and their microenvironment

Currently, most studies have addressed the cells and molecules involved in the adult bone marrow HSC microenvironment. It was shown that different types of niches for HSCs co-exist, the endosteal niche in which HSCs remain quiescence and the vascular niche, which stimulates proliferation and differentiation of HSCs. Their functions seem to be related to their location within the bone marrow and the types of cells comprising these niches. In my thesis studies, I focussed on different embryonic microenvironments and examined their roles during development. I also showed that growth fac-

tors could play different roles at different anatomical sites: IL-1 plays a role in AGM and fetal liver and acts on both HSC and cells of the microenvironment. The FGF signalling pathway may influence hematopoiesis via the AGM microenvironment.

Moreover, I show here for the first time that Runx1 transcription factor could play a role in the AGM microenvironment. Together with the results of others, it seems that Runx1 plays multiple roles, in HSCs as well as cells of the microenvironment (Figure 1). Mesenchymal and/or endothelial cells expressing Runx1 could influence HSCs by the production of growth factors and/or components of the extracellular matrix that act directly on HSCs (Figure 1 pathway A) or act additionally or alternatively via the microenvironment to produce a second wave of growth factors and/or extracellular matrix components that will act on HSCs (Figure 1 pathway B). Whether directly or indirectly, Runx1 could affect pathways that influence HSC and HPC maintenance, survival, proliferation and self-renewal. Because Runx1 is also expressed within the HSCs and HPCs, Runx1 could modulate the expression of growth factors and/or their receptors influencing the survival and proliferation of the HSCs themselves (Figure 1 pathway C). Runx1 could determine the decision between the differentiation into more committed progenitors or the self-renewal of HSCs. Modulation of expression of growth factors and/or their receptors could also affect the cross-talk between the hematopoietic cells and the cells of the microenvironment (Figure 1 pathway D). Currently, it is unknown which molecules in the cells comprising the microenvironment are regulated by Runx1. It has been suggested that multiple signalling pathways cooperate in the microenvironment. Our studies implicate two well-known signalling pathways, IL-1 and FGF pathways, both affecting the function of the embryonic hematopoietic microenvironment. So far these growth factors or their receptors are not known as target genes of Runx1. It would be interesting to determine whether Runx1 regulates IL-1 and/or FGFs (or their receptors) in the embryonic microenvironments. The stromal cell lines generated from wild type or deficient Runx1 AGM regions could provide a powerful tool to study the possible cooperation of these pathways.

Conclusions

The aim of the investigations presented here was to gain more insight into how HSC emergence and/or expansion is regulated, mainly examining the role of growth factors and transcription factors in the microenvironment. We revealed that FGF signalling induces changes in the AGM-derived microenvironment (*in vitro*) and that upon co-culture with exogenously added FGFs, HPC support is increased. Furthermore, there are indications that FGF signalling in hematopoiesis is dependent on the mouse background, as shown by the differences in *Fgfrs* expression patterns on immature haematopoietic cells. Another signalling pathway, the IL-1 pathway, was studied and found to influence the embryonic microenvironment both in AGM region and fetal liver. We found that IL-1 induced changes in the microenvironment that ultimately influenced hematopoiesis. Strikingly, IL-1 increases HPCs in the AGM and fetal liver, but only HSCs in the AGM revealing a tissue-specific effect of IL-1. Most importantly, we reveal for the first time the importance of Runx1 transcription factor in the

AGM microenvironment. This role seems highly dependent on the dose of Runx1 in this environment. Together, the studies add to our knowledge on the regulation of hematopoiesis and the role of the growth factors and transcription factors in the embryonic microenvironments.

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Chapter 7

Summary

Samenvatting voor iedereen

Summary

The adult hematopoietic system is comprised of a hierarchy of cells with the hematopoietic stem cell (HSC) at its foundation. HSCs give rise to progenitors that differentiate into mature hematopoietic cells, which perform the physiological functions of the hematopoietic system. The mature hematopoietic cells have a limited life span and need to be continuously replaced during adult life through the differentiation of HSCs. However, the pool of HSCs also has to be kept intact. The decision whether a dividing HSC differentiates towards a mature hematopoietic cell or preserves it self in an undifferentiated state is a tightly regulated process. An important player in this regulation is the so-called microenvironment (cells surrounding the HSCs).

The adult hematopoietic system resides mainly within the bone marrow microenvironment. The hematopoietic supportive microenvironment changes during the development of an organism. Within the embryo the first HSCs are generated in close association with the dorsal aorta (major blood vessel). The dorsal aorta is part of a larger structure referred to as the aorta-gonad-mesonephros (AGM) region. During later stages in development, HSCs are found in the yolk sac, placenta and fetal liver. The fetal liver will eventually harbour the HSCs until birth when they colonize the bone marrow. All these hematopoietic tissues are believed to provide a specialized microenvironment for the generation, expansion and/or maintenance of HSCs. Despite the fact that new insights into the interactions of HSCs and the bone marrow microenvironment have been revealed in the last few years, the interplay between the embryonic microenvironment and the HSCs remains under intensive investigation. To better understand how HSCs communicate with the microenvironments we studied the role of growth factors FGFs and IL-1 and the Runx1 transcription factor in the HSC supportive microenvironments provided by embryonic cell lines and embryos.

As an interesting family of growth factors, the Fibroblast Growth Factors (FGFs) have previously been implicated to play a role in the communication between the bone marrow microenvironment and HSCs. As described in Chapter 2, our studies showed that FGFs not only influence adult hematopoiesis, but are also regulators of embryonic hematopoiesis. More specifically, FGFs influence the supportive capacities of the AGM microenvironment for hematopoietic progenitors. As it is known that inbred mouse strains differ in HSC pool size as well as cycling activity, we compared the effects of FGFs on the bone marrow supportive microenvironment in six mouse strains. We showed that indeed there are strain-specific hematopoietic cell regulatory differences that could at least be partially due to the FGF signalling pathway.

A pro-inflammatory cytokine Interleukin-1 (IL-1) is involved in adult hematopoiesis. In Chapter 3 and 4, we show that IL-1 is also an important regulator of progenitors and HSCs in the mouse embryo. In the AGM and fetal liver, IL-1 affects the microenvironment. Strikingly, IL-1 increases both AGM progenitors and HSCs, whereas in fetal liver only progenitor activity is affected. Thus IL-1 acts differentially on HSCs in distinct microenvironments.

An important transcription factor in the hematopoietic system is Runx1, which was previously shown to play an intrinsic role in HSCs. Interestingly; Runx1 is also expressed in the cells of the AGM

microenvironment. To determine whether Runx1 affects the function of the microenvironment we generated stromal cell lines from AGMs wild type or deficient for Runx1 and studied their supportive capacities for progenitors and HSCs. Our results, as described in Chapter 5, show for the first time that the growth of progenitors and HSCs is dependent upon the levels of Runx1 in the AGM microenvironment.

The studies presented in this thesis add to our understanding of the role that growth factor, cytokines and transcription factors play in the communication between hematopoietic cells and the microenvironment. The challenge for future research will be to further elucidate the unique vs. overlapping roles of hematopoietic cell regulators in the adult and embryonic microenvironments.

Samenvatting voor iedereen

Het hematopoietische (bloed) systeem in een volwassene heeft een hiërarchische opbouw. Boven aan deze hiërarchie staat de zogenaamde hematopoietische stamcel en onderaan de verschillende typen rijpe (functionele) bloedcellen die verschillende functies vervullen. Deze rijpe bloedcellen hebben echter een relatief korte levensduur. Om het hematopoietische systeem in stand te houden is het dus noodzakelijk dat er een continue aanmaak van deze rijpe bloedcellen plaatsvindt door middel van het uitrijpen van de hematopoietische stamcellen. Echter, de poel van hematopoietische stamcellen moet ook intact blijven. De balans tussen het uitrijpen/differentiëren tot een functionele bloedcel en het ongedifferentieerd blijven in de hematopoietische stamcelpoel is strak gereguleerd. Een goede balans tussen het wel of niet uitrijpen van de hematopoietische stamcellen zorgt er op het juiste moment voor dat de juiste hoeveelheid en typen bloedcellen aangemaakt worden. Als deze balans goed functioneert kan het hematopoietische systeem zijn belangrijke taken, zoals zuurstoftransport of verdediging tegen ziekteverwekkers, een mensenleven lang blijven uitvoeren.

Hematopoietische stamcellen bevinden zich in een zogenaamde micro-omgeving. Deze omgeving bestaat uit allerlei typen cellen welke een belangrijke rol spelen bij het behouden van de balans tussen wel of niet uitrijpen van de stamcellen. In een volwassen mens of muis bevinden de hematopoietische stamcellen zich in het beenmerg. Deze hematopoietische stamcellen worden tijdens de embryonale ontwikkeling gevormd. Tijdens de embryonale ontwikkeling verandert de locatie van de hematopoietische stamcellen en dus ook de micro-omgeving waarin ze zich bevinden. De allereerste hematopoietische stamcellen kunnen we vinden in de nabijheid van een van de grootste bloedvaten van het embryo, genaamd de dorsale aorta. Deze dorsale aorta ligt ingebed in een weefsel wat later in de ontwikkeling de nier en geslachtsorganen vormt. Tezamen wordt deze regio de aorta-gonad-mesonephros regio genoemd, ofwel in het kort AGM regio. Vlak na het ontstaan van de hematopoietische stamcellen in de AGM verlaten ze deze regio en vinden we ze in de dooierzak, placenta en foetale lever. Uiteindelijk wordt de lever de belangrijkste plaats voor hematopoietische stamcellen in de foetus tot de geboorte. Na de geboorte migreren de hematopoietische stamcellen naar het beenmerg en blijven hier gedurende het verdere leven. Alle hematopoietische weefsels (AGM, dooierzak, placenta, lever en beenmerg) bevatten een eigen micro-omgeving. Deze micro-omgeving draagt zorg voor de vorming, vermeerdering en/of behoud van de hematopoietische stamcellen.

Hoe de communicatie tussen de hematopoietische cellen en de cellen van de micro-omgeving verloopt wordt al lange tijd bestudeerd. Om meer details hiervan te ontrafelen wordt er veelal gebruik gemaakt van cellijnen afkomstig van de verschillende hematopoietische weefsels. Deze cellijnen vormen een kunstmatige micro-omgeving welke (in een celkweek systeem) kan worden bestudeerd. Om meer inzicht te verkrijgen in de rol die de micro-omgeving speelt in de vermeerdering en/of het wel of niet uitrijpen van de hematopoietische stamcellen, hebben we onderzocht wat voor rol de groeifactoren FGFs en IL-1 en de Runx1 transcriptiefactor is in de cellen van de micro-omgeving. We hebben ons voornamelijk gericht op het bestuderen van de micro-omgevingen tijdens de embryonale ontwikkeling.

Een interessante familie van groeifactoren is de Fibroblast Groei Factor (FGF) familie. Eerdere studies hadden deze groeifactoren geïmpliceerd in de communicatie tussen hematopoietische stamcellen en de micro-omgeving van het beenmerg. Zoals beschreven in Hoofdstuk 2, laten we zien dat FGFs niet alleen in een volwassen micro-omgeving (die van het beenmerg) maar ook in een embryonale micro-omgeving (die van de AGM) een rol spelen. Het toevoegen van FGFs aan deze AGM micro-omgeving beïnvloed de manier waarop hematopoietische voorlopercellen (de afstammelingen van de stamcellen) worden ondersteund. Verder hebben we gekeken naar effecten van FGFs op de beenmerg micro-omgeving van verschillende muizen stammen. We weten al dat muizen stammen kunnen verschillen in de hoeveelheid hematopoietische stamcellen en in de activiteit van deze hematopoietische stamcellen. Onze studie laat zien dat er muisstam specifieke verschillen in de regulatie van hematopoietische cellen zijn, wat in ieder geval gedeeltelijk kan worden veroorzaakt door FGFs.

Interleukine-1 (IL-1) is een cytokine welke al eerder in het volwassen hematopoietische systeem een rol bleek te spelen. In Hoofdstuk 3 en 4 hebben we aangetoond dat IL-1 hematopoietische voorlopercellen en stamcellen kan reguleren tijdens de embryonale ontwikkeling. Behalve dat IL-1 een direct effect op de hematopoietische voorloper- en stamcellen uitoefent, kan IL-1 ook de cellen van de micro-omgeving beïnvloeden. In de AGM nemen zowel de hematopoietische voorlopercel als de stamcel in aantal toe door IL-1. Dit is in tegenstelling tot de foetale lever waar, onder invloed van IL-1, alleen de hematopoietische voorlopercel in aantal toeneemt. Hieruit kunnen we concluderen dat IL-1 verschillende effecten op hematopoietische voorloper- en stamcellen kan hebben, afhankelijk van de micro-omgeving waarin deze cellen zich bevinden.

Een belangrijke transcriptiefactor in het hematopoietische systeem is Runx1. Uit eerdere studies is gebleken dat in de hematopoietische stamcellen zelf Runx1 een belangrijke rol speelt. Als er geen Runx1 tot expressie komt tijdens de embryonale ontwikkeling, worden er geen hematopoietische stamcellen gemaakt in de AGM. Verder is gebleken dat Runx1 ook tot expressie komt in de cellen van de AGM micro-omgeving. Aangezien Runx1 in de stamcellen een zeer belangrijke rol heeft, maar ook in de cellen van de AGM micro-omgeving tot expressie komt, hebben we onderzocht wat de functie is van Runx1 in deze micro-omgeving. Zoals beschreven in Hoofdstuk 5 hebben we om deze vraag te beantwoorden cellijnen gemaakt van AGMs welke een normale en gebrekkige expressie hebben van Runx1. Deze cellijnen zijn vervolgens getest op hun ondersteunende eigenschappen voor hematopoietische voorloper- en stamcellen. Met deze studie laten we voor de eerste keer laten zien dat de vermeerdering van hematopoietische voorloper- en stamcellen afhankelijk is van de hoeveelheid Runx1 aanwezig in de AGM micro-omgeving.

Samengevat hebben de studies in dit proefschrift bijgedragen aan een beter inzicht in de rol die de verschillende micro-omgevingen kunnen spelen in de vorming, vermeerdering en het behoud van de hematopoietische stamcellen. Groeifactoren, cytokinen en transcriptiefactoren spelen hierbij een belangrijke rol. In toekomstig onderzoek zal het belangrijk zijn om de unieke en overlappende rol van hematopoietische celregulatoren in de volwassen en embryonale micro-omgevingen te bekijken.

Abbreviations

AGM region	*aorta gonad mesonephros region*
AM	*aorta-mesenchyme*
AML1	*acute myeloid leukaemia 1*
Ang-1	*angiopoietin 1*
BMC	*bone marrow cells*
BMPR1A	*bone morphogenetic protein receptor type 1A*
BL-CFC	*blast-colony forming-cell*
BrdU	*5'-bromo 2'-deoxy-uridine*
CAFC	*cobblestone-area-forming-cell*
CaR	*Ca^{2+} -sensing receptor*
CBF	*core binding factor*
CD34	*cluster of differentiation 34*
cDNA	*copy DNA*
CFU-C	*colony-forming unit-culture*
CFU-S	*colony-forming unit-spleen*
CLP	*Common lymphoid progenitorc*
CMP	*common myeloid progenitor*
CSF	*colony stimulating factor*
CXCR4	*chemokin CXC motif receptor 4*
DNA	*deoxyribonucleic acid*
E 7.5	*embryonic day 7.5*
ESC	*embryonic stem cell*
FACS	*fluorescence-activated cell sorting*
FCS	*fetal calf serum*
FGF	*fibroblast growth factor*
FGFR	*fibroblast growth factor receptor*
FL	*fetal liver*
GFP	*green fluorescent protein*
HPC	*hematopoietic progenitor cell*
HSC	*hematopoietic stem cell*
IκB	*inhibitor of kappa B*
IL-1	*interleukin 1*
IL-1R1/II	*interleukin 1 receptor 1/2*
IL-1RacP	*interleukins 1 receptor accessory protein*
JNK	*jun kinase*
LMPP	*lymphoid-primed multipotent progenitor*
LSK cells	*Lineage marker $Sca\text{-}1^{+}$ $c\text{-}kit^{+}$ cell population*
LTC-IC	*long-term culture-initiating cell*
LTR	*long-term repopulation*
MAPK	*mitogen activated protein kinase*
MMP	*matrix metalloproteinase*
MPP	*multipotent progenitor*
NFκB	*nuclear factor kappa B*
NK cell	*natural killer cell*
NMTS	*nuclear matrix targeting signal*
PAS	*para-aortic splanchnopleura*
PBS	*phosphate buffered saline*
RHD	*runt homology domain*
RT-PCR	*reverse transcriptase polymerase chain reaction*
SCF	*stem cell factor*
SCL	*stem cell leukemia*
SDF-1	*stromal derived factor 1 (Cxcl12)*
SNO	*spindle-shaped N-cadherin-expressing osteoblast*
STR	*short-term repopulation*
TAB2	*TGF beta activated kinase binding protein 2*
TNF	*tumour necrosis factor*
TPO	*thrombopoïetin*
UG	*urogenital-ridges*
VEGF	*vascular endothelial growth factor*
VEGFR2	*vascular endothelial growth factor receptor 2*

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Curriculum Vitae

Esther Haak werd geboren op 14 november 1977 te Rotterdam. In 1994, na het behalen van haar MAVO-diploma te Papendrecht, is ze begonnen aan de studie Biologische Laboratoriumtechniek aan het Zadkine College te Rotterdam. Hier behaalde ze haar MBO-diploma in 1998 waarna ze haar studie vervolgde op de Hogeschool Rotterdam & Omstreken in de richting Biochemie. Na een stage op de afdeling Neuroscience van de Erasmus Universiteit onder begeleiding van Dr. P. French en Prof. dr. De Zeeuw studeerde ze af in juli 2001. Aansluitend is ze gestart als analist op de afdeling Genetica aan de Erasmus Universiteit waar ze werkzaam was in de groep van Dr. H. Roest en Prof. dr. J.H.J. Hoeijmakers. Tijdens deze werkzaamheden vervolgde ze haar studie in het laboratorium onderwijs in de richting Medische Biologie welke werd afgerond in februari 2002.

Vanaf oktober 2002 werkte ze aan haar promotieonderzoek aan het Erasmus MC afdeling Celbiologie, onder begeleiding van Prof. dr. E.A. Dzierzak. Het onderzoek had tot doel een inzicht te verkrijgen in de communicatie tussen hematopoietische cellen en de verschillende embryonale omgevingen waarin deze cellen zich ontwikkelingen. Vanaf oktober 2006 zal zij het onderzoek naar deze communicaties in de groep van Prof.dr. E.A. Dzierzak voortzetten.