Regulation of hematopoietic stem cells during mouse development

Regulatie van hematopoietische stamcellen gedurende de ontwikkeling van de muis

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voor René

Abbreviations

AGM region	aorta-gonad-mesonephros region
AML	acute myeloid leukemia
BM	bone marrow
BMP	bone morphogenetic protein
cDNA	copy DNA
CD34	Cluster of Differentiation 34
DD RT-PCR	differential display reverse transcriptase polymerase chain reaction
DNA	deoxyribonucleic acid
Dvl	dishevelled
E10	embryonic day 10
FACS	fluorescence activated cell sorter
FL	fetal liver
GFP	green fluorescent protein
Hh	Hedgehog
HSC	hematopoietic stem cell
HPC	hematopoietic progenitor cell
IκB	Inhibitor of kappa B
IKK	IκB kinase
IAP	inhibitor of apoptosis protein
IL-1	interleukin 1
IL-1RI/ II	interleukin 1 receptor 1 /2
IL-1RAcP	interleukin 1 receptor accessory protein
IRAK	interleukin receptor associated kinase
JNK	jun kinase
kb	kilo base
kD	kilo Dalton
LEF	lymphocyte enhancing factor
LPS	lipopolysaccharide
LTRA	long-term repopulation assay
MAPK	mitogen activated protein kinase
MnSOD	manganese superoxide dismutase
MMP	matrix metalloproteinase
NFκB	nuclear factor kappa B
PBS	phosphate buffered saline
P-Sp	para-aortic splanchnopleura
RANK	receptor activator of NFκB
RHD	Rel homology domain
(m)RNA	(messenger) ribonucleic acid
SCL	stem cell leukemia
SCF	stem cell factor (kit ligand)
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TAB1/ 2/ 3	TGF beta Activated Kinase Binding protein 1/ 2/ 3
TAK1	TGF beta Activated Kinase 1
TAL	T cell acute lymphoblastic leukemia
TBS-T	Tris buffered saline with tween
TCF	T cell transcription factor
TGFβ	Transforming Growth Factor beta
TRAF6	TNF receptor associated factor
TNFα	Tumor necrosis factor alpha

Contents

Chapter 1

General Introduction

The hematopoietic system is comprised of many different cell types that fulfill important physiological functions throughout embryonic and adult stages of mouse development. As the mature blood cells have a limited life-span, the pool of blood cells needs constant replenishing. At the basis of the hematopoietic system reside the hematopoietic stem cells (HSCs). HSCs are characterized by the ability to self-renew and to produce all the different mature hematopoietic cell types[120,143,178]. These HSCs differentiate to the common lymphoid and myeloid progenitors (CLP and CMP) which in turn give rise to more lineage restricted precursor cells that then differentiate into mature hematopoietic cells (Figure 1). Mature hematopoietic cells can be subdivided into the lymphoid compartment, comprising T and B lymphocytes which form the adaptive immune (defense) system, and the myeloid compartment which is comprised of the oxygen transporting erythrocytes (red blood cells), blood clot-mediating platelets, immune response-mediating neutrophils, eosinophils and basophils and the macrophages, of which the main function is to remove dead and foreign cells. Although the mature blood cells fulfill the physiological functions of the hematopoietic system, the hematopoietic stem cells are essential within the adult hematopoietic hierarchy since these cells are responsible for the life long production of the different mature hematopoietic cells.

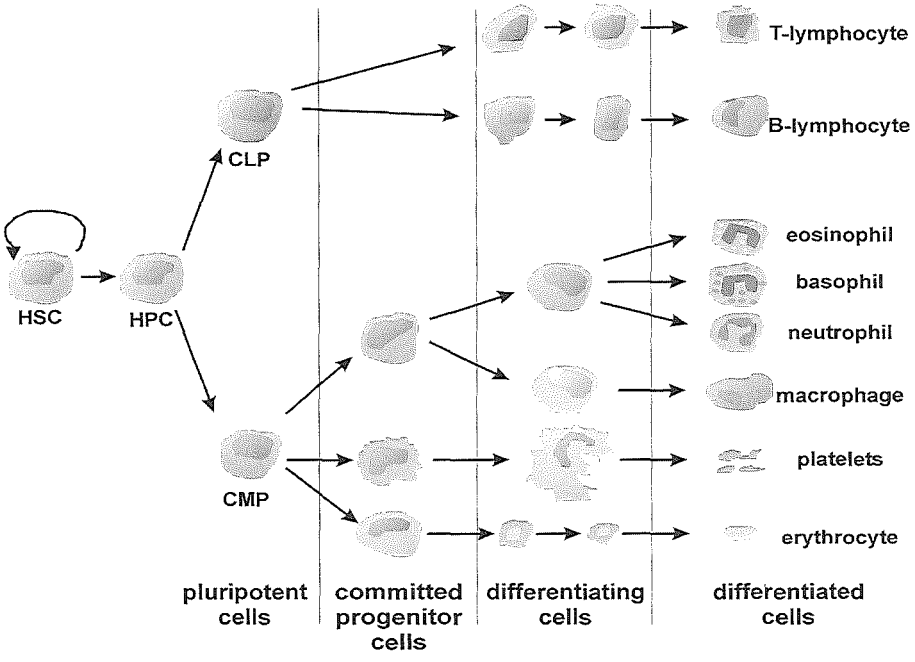

Figure 1. Schematic overview of the hematopoietic hierarchy in the adult mouse
Hematopoietic stem cells (HSC) are at the basis of the hematopoietic hierarchy and produce the pluripotent progenitors that give rise to committed progenitors which in turn differentiate into the lineage restricted mature blood cells. HPC= hematopoietic progenitor cell; CLP= common lymphoid progenitor and CMP= common myeloid progenitor. Adapted from Gilbert [72]

Characteristics of mouse hematopoietic stem cells

How to identify hematopoietic stem cells

In the adult mouse, hematopoietic stem cells (HSCs) are localized in the bone marrow where they receive signals from the micro-environment to either retain their stem cell characteristics or to proceed along the differentiation pathway of the hematopoietic hierarchy. HSCs are a rare cell type in the adult bone marrow with as few as 1 to 10 HSCs in 100.000 cells[20,79,80]. To measure HSC activity, the *in vivo* long-term repopulation assay (LTR assay) is still the gold standard[143,161]. In this assay marked donor cells are injected into an irradiated adult recipient and reconstitution of the hematopoietic system is analyzed several months post-transplantation. Limiting dilution and competitive long-term repopulation experiments can be used to determine in a more quantitative and qualitative manner the repopulation ability of HSCs within a certain cell population[80]. Besides the repopulation assay other methods are being used to identify HSCs and progenitor cells. A well known *in vivo* method is the CFU-S assay (colony forming unit of the spleen). Upon transplantation of HSCs/ progenitor cells, a fraction of these cells will migrate to the spleen and form macroscopic colonies consisting of progenitor cells and differentiated blood cells. The number of colonies correlates with the number of HSC/ progenitor cells that were originally transplanted[120,58]. Also, since HSC/ progenitor cells form hematopoietic colonies when placed in the appropriate microenvironment, several *in vitro* methods have been developed. Best known methods are the colony forming unit (CFU) assays in which HSCs/HPCs are cultured in a semi-solid medium with hematopoietic growth factors and the long term culture-initiating cell (LTC-IC) assay, where HSCs/HPCs are grown together with supportive stromal cells (reviewed in references 58, 227).

Together, these methods allow identification of the most immature cells of the hematopoietic system. The major advantage of the *in vitro* methods to identify HSCs and progenitor cells is that the environment can be controlled and manipulated. The *in vivo* LTR assay has as major advantage that true HSC activity can be determined within the physiological micro-environment. In both cases stem cell activity is measured indirectly through the presence of HSC-derived differentiated cells. Impairment either in homing capacity, proliferation or differentiation of HSCs will all result in a lack of HSC-derived mature blood cells and will be interpreted as a lack of stem cell activity, without discrimination between the different defects. The use of *in vitro* techniques to identify HSCs and progenitor cells circumvents the migration aspect of HSC homing to the correct microenvironment as is required for the *in vivo* techniques. However, the distinction between a defect in proliferation or differentiation is less easily made, particularly if the blockage occurs early in the hematopoietic hierarchy.

Markers to identify hematopoietic (stem) cells

With the methods described above the presence of HSCs and progenitor cells within a cell population can be determined, but the cellular characteristics remain unresolved. In 1891 Romanovsky discovered that upon exposure of cells to specific dyes several hematopoietic cell types can be distinguished based on the staining characteristics and the morphology of the cells[104]. The use of antibodies recognizing proteins expressed on the cell surface of hematopoietic cells allows further discrimination of hematopoietic cells, as the different cell types within the hematopoietic system have a characteristic cell-surface protein expression pattern. The subsequent technical development to separate cells stained

with antibodies coupled to a fluorescent dye by FACS (fluorescence activated cell sorting) has lead to more insight into the physiological properties of different cell types. This method has been proven very valuable for studying HSCs as they can be enriched from the bone marrow. Several protein markers for HSCs have been identified over the last years and an overview of some markers is provided in Table 1. It should be noted that despite the availability of multiple markers, it has proven difficult to obtain a pure population of HSCs, since most markers are expressed by more than one cell type. Therefore, usually a combination of markers (i.e. c-kit$^+$/CD34$^+$) is used to obtain highly enriched populations of HSCs by FACS sorting[196,212,213]. In addition to the markers recognizing HSCs, cell populations can also be divided into progenitor cell and mature differentiated cell fractions. A mixture of antibodies (i.e. CD4/8, B220, Gr1, Ter119) recognizing mature hematopoietic cells is used for selection of differentiated hematopoietic cells. The lineage marker negative cell (Lin$^-$) fraction contains the progenitor cells, while the differentiated cells reside in the lineage marker positive (Lin$^+$) fraction.

Another method to separate HSCs (and stem cells from other tissues) by FACS is based on the physiological characteristic that stem cells express multidrug resistance channel (MDR1). MDR1 allows high efflux of certain dyes such as Hoechst 33342 and Rhodamine123 from stem cells[17,26]. This method circumvents the use of membrane markers that are not exclusively expressed by HSCs and allows a high degree of HSC (side population or SP cells) enrichment[17,74,119]. However, not all HSCs are found within the side population fraction and thus rare HSCs can be lost.

In general, the most commonly used HSC enrichment protocol involves the depletion of mature lineage marker positive cells in combination with a positive selection for HSC markers. Some of the characteristics of the most frequently used HSC markers are described here. CD34, a member of the sialomucin family of glycoproteins, is expressed on hematopoietic stem and progenitor cells, vascular endothelium and embryonic fibroblasts[205,249] and is frequently used as a hematopoietic stem cell marker. During mouse development CD34 has proven to be a good marker for HSCs[196], but in the adult mouse CD34 expression seems not to correlate very well with HSC activity. In the adult mouse bone marrow most HSC activity is found in the CD34$^-$ fraction. Moreover CD34 expression on bone marrow HSCs was shown to be reversible, as CD34$^-$ cells upon culture could give rise to CD34$^+$ HSCs which loose CD34 expression upon transplantation[136,198,214]. The function of CD34 is not completely understood, but CD34 might play a role in mediating adhesion of leukocytes and hematopoietic progenitor to the vascular endothelium via the binding to selectins[205,229].

Sca-1 (Stem cell antigen-1) is also often used as a marker for HSCs in both the embryo as well as the adult mouse[40,212,225]. Sca-1 is a cell-surface glycoprotein encoded by the *Ly6A/E* gene and is expressed in HSCs, HPCs, T-and B-lymphoid and myeloid cells. Additionally, Sca-1 expression is found in the vascular endothelium and in the cortical tubules of the kidney (130,131,149 and references therein). The function of Sca-1 is poorly understood, but it was suggested that Sca-1 might play a role in cell signaling in lymphocytes, via Src family kinases (95 and references therein). Transgenic mice have been generated expressing the *lacZ* or *GFP* marker gene under the control of either the *Ly6E* or the *Ly6A* gene regulatory elements[130,149]. Analysis of these mice revealed that marker expression was found in tissues that were previously shown to express Sca-1. In transgenic mice with the *GFP* marker under the control of the *Ly6A* gene regulatory

elements all HSC activity was found in the GFP$^+$ cell population in the bone marrow and in fetal hematopoietic sites[40]. Analysis of Sca-1 (*Ly6A*) deficient mice by Ito et al. showed that mice lacking functional Sca-1 have mild thrombocytopenia, decreased numbers of colony forming cells and defects in the hematopoietic stem cell compartment as determined by competitive repopulation assays[95]. Besides the repopulation disadvantage, HSCs of Sca-1-/- mice did not engraft secondary recipients. This suggests that Sca-1 might fulfill a role either in the generation, maintenance or self-renewal of HSCs in the mouse.

Table 1. Overview of hematopoietic (stem) cell markers.

Marker /CD number	Cell types expressing the marker	Remarks	Reference
AA4.1	- HPCs in fetal liver and adult BM - vascular endothelium - lung and heart in adult	transmembrane protein	185
α11b integrin/ CD41	- HPCs in YS and AGM - megakaryocytes - platelets	cell adhesion	147,152
CD34	- HSCs/ HPCs - vascular endothelium - embryonic fibroblasts	glycoprotein	54,136,198,214, 249
CD45	- hematopoietic cells	tyrosine phosphatase	116,190
c-kit/ CD117	- HSCs/ HPCs - mast cells - testis - melanocytes - vascular endothelium	receptor tyrosine kinase	5,23
Flk-1	- HSCs in the embryo and adult - vascular endothelium	receptor tyrosine kinase	34,202,245,246
Mac-1 (αMβ2 integrin)/ CD11b	- HSCs in AGM and fetal liver - macrophages, B and T-cells	cell adhesion	116,196
PECAM / CD31	- HSCs/HPCs - T and B cells, myeloid cells - platelets - vascular endothelium - several adult tissues	glycoprotein cell adhesion	54,97,116,190
Sca-1	- HSCs/HPCs - T and B cells, myeloid cells - vascular endothelium	glycoprotein	130,131,149
Tie-2 and Tek	- HSCs - vascular endothelium	receptor tyrosine kinase	54,78,86

Another frequently used HSC marker is the tyrosine kinase receptor c-kit which binds to the ligand SCF (stem cell factor). Within the bone marrow approximately 5-10% of the cells express c-kit and HSC activity is confined to the c-kit expressing cells[89]. Also in

the midgestation AGM and liver HSC activity is confined to the c-kit expressing cells[196]. Moreover, expression of c-kit overlaps with other HSC markers, including Sca-1 and CD34[89,196]. Mice deficient for the receptor c-kit or its ligand SCF display severe anemia and defects in the HSC compartment[5,23,67]. The function and expression of c-kit and SCF will be discussed in more detail in the section regulators of HSCs.

Interestingly, the expression of certain HSC (and progenitors) markers is dependent of the developmental stage and site of hematopoiesis. For example, the markers CD41 and Mac-1/CD11b are expressed on HSCs/HPCs only during embryonic stages and not in the adult bone marrow[147,152,196]. This temporal expression could reflect a specific developmental function for these proteins. Since both these markers belong to a family of cell adhesion receptors, the expression of CD41 and Mac-1 on HSCs in the embryo might fulfill a role in migration of HSCs in the embryo. For this reason, a closer investigation of known HSC markers with an unknown function could reveal novel insights into the physiology and properties of HSCs.

Hematopoiesis and HSCs in the mouse embryo.

Localization of HSCs and hematopoietic cells in the mouse embryo

Similar to other organs during mouse development, the hematopoietic system is shaped and remodeled in a dynamic temporal and spatial manner until the end of gestation. In the adult mouse HSCs are located in the bone marrow, however during development HSCs are located from E11 onwards in the fetal liver, where their number increases dramatically[60,114,161]. Around birth HSCs migrate from the fetal liver to bone marrow where they will remain throughout the adult stages of life. Hence, during development the fetal liver is an important hematopoietic organ, although it is not the only hematopoietic organ during development (Figure 2A). The yolk sac is the site where the first hematopoietic cells in the mouse embryo arise around E7.5. These primitive, nucleated erythrocytes are formed in so-called blood islands in close association with the developing yolk sac vasculature (Figure 2B; reviewed in reference 181).

The first primitive hematopoietic cells arise in the extra-embryonic yolk sac, but more complex hematopoiesis occurs in the intra-embryonic fetal liver during later stages of development. The question arises whether the adult hematopoietic system and more importantly whether HSCs are generated in the extra-embryonic or the intra-embryonic sites during development. Around 1970 Metcalf and Moore showed that the E8 yolk sac, but not the embryo proper, has the ability to produce hematopoietic colonies both *in vitro* as well as *in vivo*. Additionally, injection of E11 yolk sac cells into lethally irradiated recipients resulted in hematopoietic reconstitution by the donor cells 30 days post-transplantation. Based on these results it was concluded that the extra-embryonic yolk sac and not the embryo proper produces hematopoietic stem cells that migrate to the fetal liver during later stages of mouse development[156].

In 1975 the hypothesis on the yolk sac origin of the adult hematopoieitic system during development was challenged by the studies of Dieterlen-Lievre and Turpen in the avian and amphibian model systems respectively. Grafting experiments with chick-quail chimeric embryos showed that the adult hematopoietic system is derived from an intra-embryonic source and that there is no contribution by the yolk sac[47]. Experiments with amphibians revealed that an intra-embryonic source contributes to a major extent to the

adult hematopoietic system. Also a minor contribution by the yolk sac to the adult hematopoietic system was observed[106,224]. In agreement with the non-mammalian vertebrate models, careful mapping studies and LTR-assay studies in the mouse embryo showed that the first adult-repopulating HSCs become detectable within the embryo proper in a region referred to as AGM (aorta-gonad-mesonephros) from E10.5 onwards (Figure 2C)[142,161]. From E11 onwards HSC activity becomes detectable in the circulation, the yolk sac and the fetal liver[114,142,161]. Moreover, when E10 yolk sac and AGM tissues were cultured in an organ culture system and analyzed by LTR-assays it was shown that the AGM, but not the yolk sac, autonomously generates HSCs[142]. The use of this organ culture system excludes the possibility that HSCs from the yolk sac seed the AGM region via the circulation and therefore these data demonstrate that the AGM is the first site in the embryo where HSCs are autonomously generated. A closer examination of the AGM revealed that these adult-repopulating HSCs arise in aorta subregion[42]. At the same time HSCs can also be found in the vitelline and umbilical arteries[42]. These blood vessels are derived from intra-embryonic tissue and connect the embryo proper with the yolk sac and the placenta.

Recent studies in several species, including human and *Xenopus* also revealed the existence of an intra-embryonic source of multipotent hematopoietic (stem) cells[219,35]. Fate-mapping studies showed that in *Xenopus* the yolk sac equivalent (ventral blood islands) and the AGM equivalent (dorsal lateral plate) arise from different cells (blastomeres). The separate origins of these tissues and the finding that definitive blood is produced from an intra-embryonic source in the *Xenopus* embryo, correlates with the findings that HSCs are derived from an intra-embryonic source. Nevertheless the debate on the origin of HSCs in the mouse embryo is still ongoing and recent studies have again challenged the idea that HSCs originate from an intra-embryonic source[247]. Injection of E9 yolk sac or para-aortic splanchnopleura (P-Sp)[a] cells into the liver of HSC-ablated neonatal recipients resulted in repopulation of the hematopoietic system for a prolonged period of time. These experiments suggest that the yolk sac does contain neonatal LTR-HSCs, but that these cells upon injection into adult recipients fail to repopulate the hematopoietic system. It was proposed that E9 yolk sac HSCs have not gone through the necessary maturation processes (i.e. expression of the correct cell adhesion receptors) in order to engraft the bone marrow of adult recipients, and are therefore not detected in LTR-assays in adult recipients. To date, it remains unclear whether these neonatal LTR-HSCs are autonomously generated in the yolk sac, AGM or in both tissues. However, these studies do indicate that besides adult LTR-HSCs, neonatal LTR-HSCs exist. While both possess the similar physiological properties of long-term reconstitution of the hematopoietic system, they differ greatly in their properties of homing and/or engraftment.

[a] The para-aortic splanchnopleura (P-Sp) is the precursor tissue of the AGM region

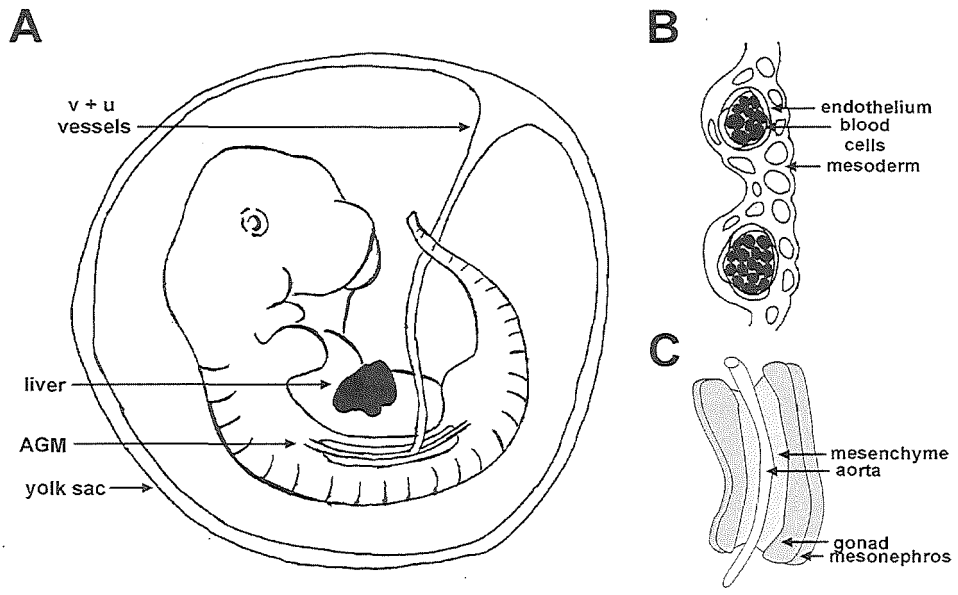

Figure 2. Schematic overview of hematopoietic tissues during mouse midgestation development. *(A) Schematic overview of a midgestation mouse embryo. The yolk sac is the first hematopoietic site in the embryo and primitive erythrocytes can be detected in the yolk sac from E7.5 onwards. The AGM region and the vitelline and umbilical (v+u) vessels are the first sites were adult repopulating hematopoietic stem cell activity can be detected and the fetal liver is the main hematopoietic organ from E12 onwards until the end of gestation (adapted from Dzierzak et al.[58]). (B) Schematic representation of a blood island of the yolk sac with primitive erythrocytes surrounded by the vascular endothelium of the yolk sac vessels (adapted from Dzierzak[57]). (C) Schematic representation of the AGM region with the dorsal aorta in the middle surrounded by the primordial gonads and mesonephros (adapted from De Bruijn et al.[42])*

The fact that prior to the emergence of detectable HSC activity in the embryo differentiated hematopoietic cells and hematopoietic precursor cells can be found both in the embryo and the yolk sac[38,41,172,180] raises another puzzling issue. One explanation could be that the hematopoietic hierarchy as known in the adult, in which HSCs give rise to mature blood cells, does not exist in the embryo. This model would be consistent with the idea that the hematopoietic system, as other organs, is generated and shaped during development and reflects the needs of the embryo during the different stages of development. A known example of organ remodeling in the embryo is the development of the kidney. The embryonic kidney during midgestation exists of the mesonephros which degenerates, after which the adult kidney is independently generated from the metanephros. Another theoretical possibility could be that HSCs from the earliest stages of development are producing all different hematopoietic progenitors, consistent with the adult hematopoietic hierarchy, but that the currently available assays fail to detect this HSC activity at early embryonic stages. Thus, due to the *in utero* development of mammals and the inability to perform embryo-grafting experiments, the exact relationship between the different hematopoietic cells, precursors and HSCs found in the mammalian embryo remains unresolved to date.

Lineage relationship between hematopoietic and vascular endothelial cells

The hematopoietic system is thought to be derived from the mesodermal lineage during development. Indeed, the close relationship between the development of the hematopoietic system and the blood vessel endothelial cells raised the idea that HSCs and endothelial cells are derived from a common mesodermal precursor, the so-called hemangioblast. The first supporting evidence for this theory comes from the observation was that primitive hematopoietic cells develop in close association with the vascular endothelium[195]. In the yolk sac, primitive erythrocytes are formed within the developing blood vessels in blood islands and similarly in the intra-embryonic AGM region HSCs arise close to or within the endothelium of the dorsal aorta[40,42,181]. Clusters at the ventral aspect of the dorsal aorta have been detected in embryos of several species, ranging from fish to human[70,100,168,218]. These clusters are believed to contain HSCs based on the expression of hematopoietic (stem) cell markers, such as CD45 and c-kit[168,169,218]. Moreover, HSCs were shown to express a number of markers, such as CD34, Flk-1, Tie-2, PECAM-1 and Tek that are also expressed by vascular endothelial cells[78,87,115,168,249]. Recently, a study with transgenic mice expressing *GFP* under the control of the promoter of the HSC marker Sca-1 showed that HSCs are GFP^+ and that GFP expression was restricted to the endothelium of the dorsal aorta in the AGM[40]. Altogether these studies show that HSCs not only share the expression of several markers with endothelial cells but that HSCs localize during a defined window of developmental time to the endothelium of the dorsal aorta.

The idea of a common precursor is further strengthened by the results of several gene targeting studies in mice. Generation of mice deficient for the Flk-1 tyrosine kinase receptor or the transcription factors Lmo2 and SCL/tal1 results in an embryonic lethal phenotype with defects in both the vascular endothelium as well as in the hematopoietic cell compartment[12,193,201,202,233,241]. Flk-1 expression has been observed in both the endothelial cells and the intravascular clusters of the aorta in the P-Sp/ AGM region and studies with chimeric mice revealed that *Flk-1* deficient ES cells do not contribute to the vascular endothelium or the primitive and definitive hematopoietic cell compartment[201,202].

In addition, studies by Choi et al.[34] showed that the cultures of ES cells to embryoid bodies gave rise to a transitional cell type (the blast-colony forming cell) that can differentiate to both endothelial and hematopoietic cell lineages. The embryoid body culture technique has previously been shown to mimic the development of yolk sac hematopoiesis and might therefore reflect the *in vivo* developmental process [107,211,237]. Altogether these data strongly suggest a common origin for the endothelial and hematopoietic cell lineages in the embryo and the studies from Choi et al. do provide evidence that *in vitro* a common precursor for endothelial and hematopoietic cells does exist. Nevertheless, formal proof that indeed one precursor cell gives rise to both endothelial and hematopoietic cells within the embryo is still lacking.

Regulators of hematopoiesis

For both the generation as well as the maintenance of the hematopoietic system the continuous input of signals from the environment needs to be translated into a cellular response. In the adult hematopoietic system a constant production of mature blood cells is required to refill the pool of mature blood. During development the production of erythrocytes is required to ensure oxygen supply to the developing embryo.

HSC proliferation, differentiation and survival.

The regulation of the cellular processes of HSCs requires tight control, as either excessive proliferation or cell death (apoptosis) will result in too many or too few hematopoietic cells. Similarly, tight control over the differentiation and self-renewal of HSCs is essential to maintain the appropriate balance of hematopoietic progenitors and mature blood cells and to protect the HSC pool for life long hematopoiesis. Several studies examined these properties of HSCs in the adult bone marrow and the results of these studies imply that HSCs in the adult are mainly quiescent, and enter cell cycle at a low frequency[1,33]. In contrast, during development the number of HSCs within the embryo increases dramatically[60,114], suggesting that HSCs are not quiescent, but highly proliferative. Alternatively, the increases in HSC number during development might be due to the differentiation of HSCs from hemangioblasts or from hemogenic endothelial cells. Nevertheless, this difference in regulation of HSC numbers most likely involves different signals in the embryo as compared to the adult or similar signals triggering a different response depending on the micro-environment. To more specifically describe the regulators of hematopoiesis the following sections will focus on apoptosis, developmental patterning factors, growth factors and transcription factors. As the number of hematopoietic regulators is still rapidly expanding, the following sections will highlight only the most important regulators related to the studies presented in this thesis.

HSC survival and apoptosis

Inherent to cell differentiation and proliferation is the decision for cells to life or die. In tissue remodeling during development and in tissue homeostasis throughout life programmed cell death/apoptosis is an essential and highly conserved mechanism which is counteracted by anti-apoptotic signals to ensure cell survival. The key players of apoptotic processes are conserved between several species and homologous proteins between mammals and the worm *C.elegans* have been identified[61,144]. The executioners of apoptosis belong to a family of cysteine proteases, named caspases, and are responsible for the degradation of the cellular organelles, the nucleus and the fragmentation of the DNA, which are the hallmarks of apoptosis[36,84]. Caspases are produced as pro-enzymes and their activity is under regulation of several adapter proteins which in turn are under regulation of members of the Bcl-2 protein family[3]. The Bcl-2 protein family consists of three subfamilies, the anti-apoptotic Bcl-2 subfamily and the pro-apoptotic Bax and BH3-only subfamilies[3]. One of the proposed mechanisms by which Bcl-2 family members may regulate apoptosis is through cytochrome c release from the mitochondria. Based on several biochemical and cellular findings it has been proposed that pro-and anti-apoptotic proteins counteract each other's function via protein-protein interactions (reviewed in 3,22).

To date it is unclear what role apoptosis exactly plays under normal physiological circumstances in the bone marrow HSC population. However, it is clear that under stressful conditions, such as exposure to irradiation, a large amount of HSCs will enter the apoptotic pathway. Overexpression of anti-apoptotic genes, for example *Bcl-2*, within the HSC compartment will result in increased viability, which is most likely due to diminished HSC apoptosis[52,53,171]. However, also in steady state conditions apoptosis might play a role in maintenance and regulation of HSCs numbers. Several studies have shown that overexpression of the anti-apoptotic *Bcl-2* gene within the HSC compartment results in a higher frequency of HSCs as determined by LTR-activity and an increased number of c-kit$^+$

cells in the bone marrow as determined by FACS analysis[52,177]. A remaining question is what signals are involved in triggering apoptosis in HSCs during normal physiology.

The regulation of the cellular processes of HSCs, such as proliferation and apoptosis, is certainly influenced by the micro-environmental cues. Both in the embryo and the adult, HSCs are surrounded by stromal cells, which will provide regulatory signals for the HSCs. To date, several regulatory signals have been identified, including morphogenetic/patterning factors and hematopoietic growth factors, but the interplay between these signals remains unclear. Despite numerous attempts to culture HSCs *ex vivo* in the presence of known hematopoietic growth factors, it is still not possible to maintain HSC activity over a prolonged period of time. However, promising new studies by de Haan et al. revealed that addition of fibroblast growth factor-1 (FGF-1) to bone marrow cells induces expansion of HSCs *ex vivo*[43]. Nevertheless, it remains unclear whether the expansion of HSCs in these cultures is induced by FGF-1 alone or whether FGF-1 acts in concert with other growth factors produced by accessory cells in these cultures. The finding that *Fgfr1*-/- ES cells are impaired in their ability to form hematopoietic cells does suggest that FGFs play a role in hematopoietic development[62]. To mimic more closely the *in vivo* situation, HSCs are often cultured in the presence of accessory or stromal cells. In contrast to the cultures with hematopoietic growth factors, the presence of stromal cells allows maintenance and, in some cases, expansion of HSC activity *ex vivo*[166,175], indicating that stromal cells provide essential signals for HSC survival and proliferation. Several explanations for the role of support by stromal cells on hematopoietic (stem) cells have been suggested, including direct cell-cell contact that provides signals for survival, proliferation or differentiation. Additionally, secretion of growth or inductive signals by stromal cells could regulate hematopoietic cell behavior. Most likely it is a combination of both cell-cell contacts and a multitude of secreted factors that provide the necessary signals for the hematopoietic cell survival and proliferation.

Developmental signaling pathways, Patterning Factors and Hematopoiesis

During development several morphogenetic signals are required for the correct spatial and temporal formation of tissues and organs. Members of the transforming growth factor β (TGFβ), Wnt and Hedgehog families were reported to play such roles during development (reviewed in 29,90,135,141,239). Morphogenetic or patterning factors are often expressed in a gradient along which several cell types are exposed to different amounts of the morphogen. Both the concentration as well as the duration of the exposure of cells to a morphogen determines the impact of the signal. Morhogenetic/patterning factors can therefore have different effects on different cell types depending on their position with respect to the source of the morphogen. Morphogens often act as priming signals that allow cells to become responsive to other instructive signals[69,77]. As the development of HSCs within the embryo also occurs in both a specific temporal and spatial manner, several studies have addressed the role of instructive signaling pathways in the induction of HSCs.

Hedgehog proteins and hematopoiesis

The hedgehog proteins Sonic and Indian hedgehog (SHH and IHH), were shown to have instructive properties during early mouse hematopoietic development with regard to the induction of hematopoietic and vascular cell fate [18,56]. Moreover, expression of IHH was

detected in the yolk sac and shown to be an important regulator of yolk sac blood island formation and vascular remodeling in the embryo proper[11,28]. Also in the adult, SHH plays a role in inducing proliferation of human hematopoietic progenitors cells[18]. The ligand SHH, the receptors patched and smoothened and the transcription factors Gli-1, 2 and 3 are expressed in primitive hematopoietic cells and blood vessel endothelial cells[18]. Interestingly, several of these studies implicated a role for Bone morphogenetic protein -4 (BMP-4) as mediator of these effects, as BMP-4 expression was upregulated by hedgehog proteins. Moreover, the BMP inhibitor, noggin, could block the proliferation of hematopoietic progenitor cells induced by SHH[18,56]. In the mouse embryo, BMP-4, a member of the TGFβ family, had been already suggested to play a role in mesoderm formation and more specifically in the development of both hematopoietic and vascular systems[238]. Altogether, these data suggest that hedgehog proteins, possibly via BMP-4 regulation, might indeed play a role in the induction or regulation of HSCs.

TGFβ family members and hematopoiesis

Several studies have implicated BMP-4 as an important regulator of hematopoiesis in the embryo. Loss of function studies in the mouse revealed that *Bmp-4* deficient embryos die early in development (between E6.5-9.5) due to impaired mesoderm formation[238]. However, careful examination of these embryos also revealed a reduced numbers of red blood cells in the yolk sac and the embryo proper[238]. *In vitro* studies with mouse embryonic stem cells showed that BMP-4 could increase the amount of hematopoietic cells formed in these cultures[103]. In addition, BMP-2, 4 and 7 were shown to allow maintenance or to induce the proliferation of mouse bone marrow HPCs[19]. Interestingly, BMP-4 expression has been observed at the ventral aspect of the dorsal aorta in the human AGM region underlying intra-aortic hematopoietic clusters believed to harbor the HSCs[134]. These data suggest a role for BMP-4 in the formation of these hematopoietic clusters or the generation of HSCs in the embryo and support the idea that BMP-4 might be a true regulator of HSCs during development.

Besides BMPs, other members of the TGFβ family have been implicated to play a role in hematopoiesis. TGFβ1 can inhibit the proliferation of endothelial and hematopoietic (progenitor) cells[25,207]. Studies addressing the role of TGFβ during development showed that 50% of the homozygous *Tgfβ1* null embryos die around E10.5 due to defects in the hematopoietic and vascular systems[46]. Embryos that do not express the TGFβ type 1 receptor die between E10.5-11.5 with defects in the vascular system, but no obvious defects in yolk sac hematopoiesis[117]. Interestingly, Endoglin, an accessory receptor for TGFβ[30,135], was shown to be a marker for bone marrow HSCs in the mouse, again indicative for a role of TGFβ in HSC regulation[31].

Wnt signaling and hematopoiesis

Wnt genes encode secreted glycoproteins that act as ligands in a highly conserved developmental signaling pathway. A large number of homologous genes have been identified in several species ranging from Drosophila to human. The key player in the canonical Wnt signaling pathway is the protein β-catenin. Upon binding of Wnt to the Frizzled (Fz) receptor degradation of β-catenin is inhibited, resulting in accumulation and translocation of β-catenin to the nucleus, where it interacts with transcription factors of the TCF/LEF family and induces activation of target genes (reviewed in 29,150,239). Besides

this canonical Wnt signaling pathway, β-catenin independent signaling has also been reported[92,183]. Several members of the Wnt family were shown to affect hematopoiesis in several species. In the mouse, Wnt5a and 10b as well as several Wnt receptors are expressed in the yolk sac and fetal liver. Both Wnt5A and 10b stimulate expansion of fetal liver progenitor cells[6]. Also in humans, Wnt2b, 5a and 10b increase the number of progenitor cells *in vitro*[226]. A more recent study revealed that Wnt5A does not affect the proliferation rate of human HSCs *in vitro*, but does enhance *in vivo* engraftment of these cells[163]. Loss of function studies in mice show that Wnt5A is required for the morphogenesis of the limb and other structures. *Wnt5A* deficient mice die shortly after birth. However, the fact that no obvious defects were detected in the hematopoietic system[244] suggests either that Wnt5A does not play an essential role in the regulation of hematopoiesis during mouse development or more likely that there is functional redundancy with other Wnt family members. Based on the expression of Wnt proteins, Wnt receptors and signaling components in hematopoietic tissues, including the yolk sac, fetal liver and adult BM, together with the effects of Wnt proteins on hematopoietic progenitor cells *in vitro,* it is likely that Wnt proteins are involved in HSC regulation. Their exact role in HSC regulation *in vivo* needs clarification in further studies.

Taken together, these studies underline the importance of instructive signals by Hegdehog, TGFβ and Wnt family members in the induction and regulation of the hematopoietic system during development. Interestingly, several of these studies indicate that these instructive signals often act on both the development of the vascular and hematopoietic system, consistent with the findings that the vascular system and the hematopoietic system develop in close association. Besides the effects of single patterning factors on hematopoietic (stem) cells, these patterning factors might act in concert to regulate hematopoietic development as several studies have shown cross-talk between these different patterning factors and their signaling components.

Growth factors and cytokines and hematopoiesis

Stem cell factor (SCF) and the c-kit receptor

The natural occurring white spotting (W) mutation in mice was first recognized in 1908 and most homozygous mutant mice die perinatally due to severe anemia. Surviving mice are sterile and have coat pigmentation defects[167]. The finding that mutations at the *Steel* (*Sl*) locus resulted in a similar phenotype, suggested a relationship between these two loci. Further investigations revealed that the *W* locus encodes for a receptor tyrosine kinase, named c-kit and that the *Sl* locus encodes the receptor ligand, named stem cell factor (SCF)[b] [5,67,23].

Expression of c-kit was detected in the hematopoietic system, the brain (hippocampus and cerebellum), the developing digestive tract, vascular and breast endothelial cells, renal tubules, melanocytes in the skin and in the gonads[5,67]. Within the hematopoietic system c-kit expression was found in several mature cell types, HSCs and progenitor cells[196,23]. During the differentiation of progenitor cells along the several hematopoietic lineages c-kit expression is down-regulated and studies with blocking antibodies indicate that c-kit is required for myelopoiesis, but not for lymphopoiesis[5].

[b] SCF is also known as Kit Ligand (KL), mast cell growth factor (MGF) or steel factor.

The ligand for the c-kit receptor is an 18.5 kD protein named SCF and alternative splicing results in either a soluble or membrane bound protein isoform. SCF expression is observed in BM stromal cells, fibroblasts, endothelial cells, keratinocytes, gut epithelial cells, the thymus, embryonic brain and spinal cord and at low levels in the circulation[c] [5,23]. Within the hematopoietic system SCF acts at multiple levels within the hematopoietic hierarchy and regulates several processes, including proliferation, cell survival, differentiation and functional activation. Moreover, SCF synergizes with several cytokines, including IL-1/3/6 to induce expansion of (human) BM progenitor cells[227,5,23]. This expansion was suggested to be caused by induction of cell cycle progression[23].

Besides its role in proliferation and cell survival, SCF is an important regulator of cell adhesion, migration and mobilization of hematopoietic cells. Injection of SCF in mice results in mobilization of HSCs from the BM to the peripheral blood[5]. Interestingly, it was shown recently that cytokine induced matrix metalloproteinase 9 (MMP-9) activation mediates the release of soluble kit ligand in the BM resulting in the cell cycle entry and mobilization of HSCs to the peripheral blood[82]. Additionally, SCF is a chemo-attractant for mast cells and progenitor cells and *in vitro* studies showed that SCF increases the avidity of the β1 integrins VLA-4 and VLA-5 of $CD34^+$ BM cells, resulting in increased adhesion to fibronectin[5]. Besides the effect of SCF on the expression/regulation of adhesion receptors, the membrane bound isoform of SCF is believed to increase adhesion of hematopoietic cells via the interaction with the c-kit receptor.

Interleukin 1 and its role as a hematopoietic regulator

Interleukin 1 (IL-1) is part of the large family of cytokines, which are low molecular weight proteins that control the development of hematopoietic cells and regulate immune responses. The cytokine family includes the interleukins, colony-stimulating factors and tumor necrosis factors[83]. Cytokines can function as chemo-attractants, growth and/or differentiation factors. In 1984 the first two interleukin genes, *IL-1*α and *IL-1*β, were cloned and since their discovery a vast number of studies have addressed the role of IL-1 in normal physiology and during pathogenesis. IL-1 plays a central role within the innate immune system and as a mediator of inflammation processes. Besides its involvement in innate immunity and inflammation, IL-1 has also implicated as a regulator of adult mouse and human bone marrow hematopoietic stem and progenitor cells. Functional studies show that IL-1 has a radio-protective effect on HSCs and progenitor cells[164,176,250] and might also be involved in HSC and progenitor cell adhesion and migration[64,157]. Furthermore, IL-1 can upregulate the expression of several cytokines, including IL-1/3/6, GM-CSF and SCF and their receptors, as well as molecules involved in hematopoietic cell adhesion and migration[49].

IL-1 in combination with other hematopoietic growth factors can induce expansion of hematopoietic progenitor and myeloid precursor cells[24,63,81]. In contrast, other studies showed that IL-1 itself had no proliferative effect and that IL-1 in combination with other cytokines induced differentiation instead of expansion of hematopoietic progenitor cells or even resulted in complete abrogation of HSC activity[98,248]. As these studies were performed with different populations of bone marrow cells, different amounts and combinations of

[c] in normal human serum at approximately 3 ng/ml

(hematopoietic) growth factors, it is difficult to compare these results. Nevertheless, the fact that in some studies IL-1 has a positive effect on the proliferation of hematopoietic stem/ progenitor cells, together with the reported expression of IL-1 and the IL-1 receptor in hematopoietic stem/ progenitors cells[96,132,139], suggests that (under certain circumstances) IL-1 is a positive regulator of adult HSCs and hematopoietic progenitor cells.

By gene targeting, mice deficient for several components of the IL-1 signaling cascade have been generated, including the *Il-1rI, Il-1α* and *Il-1β*, and the intracellular signaling mediators *Myd88* and *Irak-4*[2,73,85,215]. Mice defective for IL-1 signaling components display impaired IL-1 signaling and impaired immune responses, including lymphocyte activation and fever induction. Apart from these defects, the mice develop normally and are relatively healthy. Most likely, due to functional redundancy with IL-18, Toll, RANK and other related molecules, many effects of IL-1 signaling deficiency are masked[51,112,204].

Regulation and expression of IL-1 and the IL-1 receptors

Within the IL-1 receptor and ligand system there are multiple levels of regulation. The ligands IL-1α and β are regulated at both the transcriptional and translational level. Several studies have reported that increased *Il-1* mRNA expression did not result in increased protein expression[49]. Hence, the second level of regulation is post-translational as both IL-1α and IL-1β are produced as precursor proteins of 31 kD. Proteolytic cleavage of the precursor proteins is mediated by ICE (interleukin converting enzyme; also known as caspase 1) and results in mature 17kD IL-1 proteins. Although no obvious difference in the biological activity between IL-1α and β has been observed in several studies, a striking difference between these isoforms is the finding that in humans IL-1α is normally not secreted, while IL-1β is usually secreted and can be detected in the circulation. Moreover, it was shown that IL-1α bound to the IL-1RI can be found in the nucleus where it might act as a transcription regulator[39,154]. Besides the biologically active IL-1α and β isoforms, an IL-1 antagonizing protein, named IL-1Ra, exists which can bind to the IL-1 receptor, thereby preventing IL-1 binding and signaling. Although the amino acid similarity between IL-1α, IL-1β and IL-1Ra only ranges from 18-22%, the three dimensional protein structure is highly similar [48]. The main source of IL-1 is monocytic cells, which produce and secrete IL-1 upon a large variety of stimuli, including microbal inducers, stress factors, cytokines and extracellular matrix components. Maximal *IL-1β* gene expression in monocytes requires the transcription factor Spi-1/PU.1[110]. *IL-1* can also be induced by several neuronal factors and expression was detected in the brain[49]. IL-1Ra is expressed by several hematopoietic cells, endothelial and epithelial cells and fibroblasts[4,49].

The IL-1 receptor is composed of two subunits, the IL-1R1 and the IL-1RAcP (receptor associating protein). Upon binding of IL-1 to the IL-1R1, IL-1RAcP is recruited to the complex to stabilize the binding of IL-1 and to mediate recruitment of signaling proteins and thereby activating the signaling cascade[55]. The third IL-1 receptor, named IL-1RII has a very short 29 amino acid intracellular tail and lacks a signal propagating domain[55]. Biochemical studies showed that the IL-1RII lacks signaling properties and therefore acts as a decoy receptor[37,55,140,206]. Binding of IL-1 to the decoy IL-1RII therefore fulfills a function in regulating the amount of biologically active IL-1. Besides the inhibiting properties of the IL-1RII and transcriptional regulation, the IL-1RI and RII can be proteolytically cleaved resulting in soluble receptors with the ability to bind and

sequester IL-1[55,179,210]. The IL-1RI is 80kD and is expressed on endothelial cells, smooth muscle cells, epithelial cells, hepatocytes, fibroblasts, keratinocytes and several hematopoietic cell types. The number of IL-1 receptors expressed on the cell surface of primary cells is generally low (usually less than 200 receptors per cell). The expression pattern of IL-1RAcP is similar to that of IL-1RI and highest expression levels are found in the brain[49,55]. The IL-1RII is 60kD and its expression is more restricted than the IL-1R1 [55,206]. IL-1RII is highly expressed in myeloid and lymphoid cells and at lower levels in keratinocytes and liver cells[140]. IL-1RII binds with a very high affinity to IL-1β and with much lower affinity IL-1α[49,140].

Interleukin-1 signaling

The IL-1 receptor belongs to the IL-1/ Toll-like receptor superfamily which consists of a large number of receptors which all contain an intracellular TIR (Toll-IL-1 Receptor) domain. Within the TIR domain 3 conserved regions have been identified which are highly conserved from Drosophila to mammals and region 2 and 3 have been suggested to play a crucial role in signal transduction[55,209]. As reviewed, several signaling cascades are activated by IL-1R stimulation, including the Ras-Raf-p42/44 MAPK pathway, p38 MAPK signaling and phospholipase A2 (PLA2) signaling. The best characterized signaling pathways activated by IL-1 are the Jun kinase (JNK) pathway and the NFκB signaling cascade[49,50,55,204]. IRAK kinases (especially IRAK-4) were shown to be a crucial components of IL-1 signaling[101,215]. Interestingly, IRAK-1 was reported to translocate to the nucleus upon IL-1 stimulation, similar as reported for the IL-1R itself [21,39]. The NFκB signaling cascade which is activated by IL-1, is highly conserved within the innate immune system as shown in Figure 3. Within the IL-1 receptor signaling cascade the kinase TAK1 and the regulatory TAK1 binding proteins (TABs) play an important role in the activation of JNK and NFκB.

TGF-β activated kinase 1 (TAK1) and TAK1-binding (TAB) proteins

Transforming growth factor β activated kinase 1 (TAK1) was originally identified in a screening for proteins involved in TGF-β signaling[243]. In the adult mouse TAK1 is expressed ubiquitously, with highest expression levels in thymus and brain[243]. Analysis of TAK1 protein expression during mouse development revealed that at E10.5 the highest TAK1 expression is in the spinal cord and the brain. From E12.5 onwards high levels of TAK1 expression were observed in the gut epithelium and the meta-and mesonephros (kidney anlagen). Also in the testis, lung and pancreas TAK1 expression was detected at E14.5[99]. The human *TAK1* gene is located on chromosome 6q16.1-3 and contains 17 exons and mouse *Tak1* is located on chromosome 4. Through alternative splicing of exon 12 and 16, four different splice isoforms are generated, resulting in four proteins that differ in their C-terminus. It is unclear what the function of these different isoforms is, although it was suggested that interaction of TAK1 with TAK1 binding proteins might be altered as this interaction is mediated via the C-terminus of TAK1[111].

Soon after the identification of TAK1, the identification of two TAK1 binding proteins, TAK1 binding protein1 and 2 (TAB1 and 2[d]) was reported[208]. Human *TAB1* is

[d] TAK1 binding proteins are also referred to as map3k7ip (map3k7 interacting proteins).

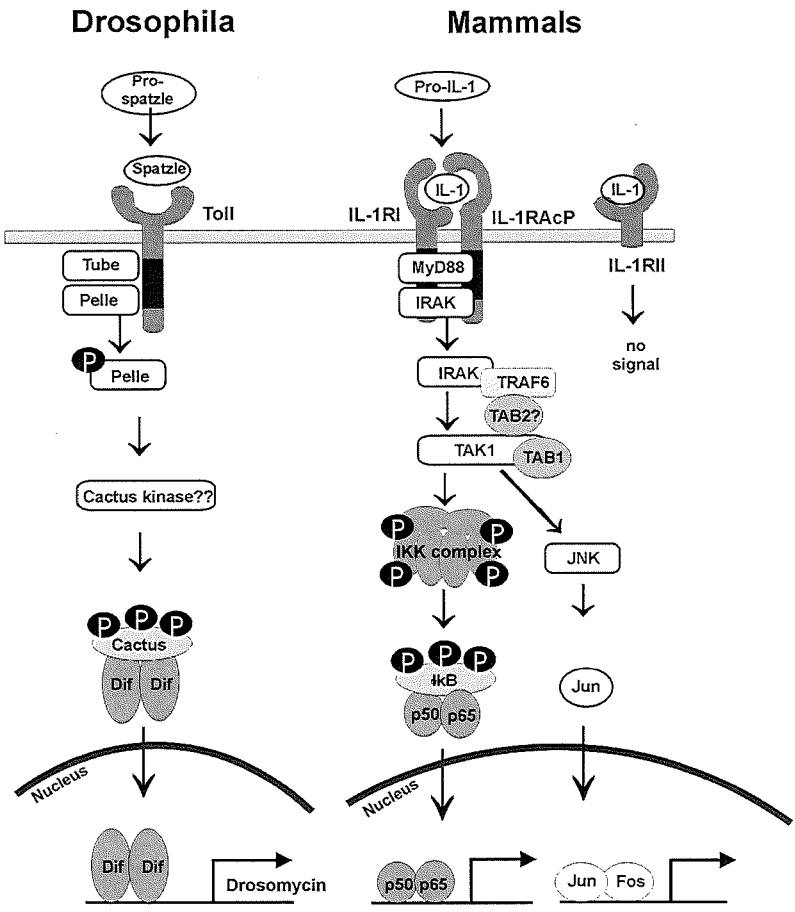

Figure 3. Signaling cascade of the Drosophila Toll and mammalian IL-1 receptor.
Signaling via the Toll/IL-1 receptor is a highly conserved pathway between Drosophila and mammals. In Drosophila the ligand pro-Spätzle needs to be proteolytically processed prior to binding to the Toll receptor, which upon binding Spätzle recruits the adapter Tube and the kinase Pelle. Upon phosphorylation and activation of Pelle, downstream signaling is propagated via to date unknown kinases towards Cactus which in turn gets phosphorylated and targeted for degradation. Upon Cactus degradation the transcription factor Dif is released and will translocate to the nucleus and activate target genes. In mammals pro-IL-1 is proteolytically processed prior to binding to the IL-1 receptor. Upon IL-1 interaction with the IL-1RI, the accessory receptor IL-1RAcP is recruited and allows interaction of the adapter protein MyD88 and the kinase IRAK with the receptor. Via the adapter proteins TRAF6, TAB1 and possibly TAB2 and the kinase TAK1 the signal is propagated to the kinase JNK which activates gene activation via the transcription factor c-jun. The signaling cascade also relays a signal to the IκB kinase complex, which will phosphorylate IκB and target it for degradation. The transcription factor NFκB is released and allowed to translocate to the nucleus in order to activate target genes. Upon binding of IL-1 to the IL-1RII no signal is propagated as the IL-1RII lacks an intracellular TIR domain which is required for recruitment of signaling proteins. The IL-1RII therefore is regarded as a decoy receptor. (Adapted from Silverman [204]).

located on chromosme 22q13 and contains 13 exons, mouse *Tab1* is localized on chromosome 15. It was shown that TAB1 is ubiquitously expressed and constitutively associated with TAK1. As overexpression of TAB1 resulted in enhanced TAK1 kinase activity, it was proposed that TAB1 is a positive regulator of TAK1. Later it was shown that a conserved C-terminal α-helix motif in TAB1 is involved in the binding and activation of TAK1[178]. Further analysis by Ge et al. revealed that TAB1 is involved in the autophosphorylation of TAK1[71]. Apart from Northern blotting and RT-PCR RNA expression analysis, the expression pattern of *Tab1* in the adult and during embryonic stages has to date not been further determined. However, *Tab1* deficient mice die around the end of gestation due to abnormal lung and cardiovascular development, suggesting an important role for TAB1 in heart and lung development[111]. Interestingly, TAK1 was implicated as an mediator of BMP signaling required for cardiomyocyte differentiation[158] and TAK1 overexpression in transgenic mice induced heart failure[255].

Both TAK1 and TAB1 are highly conserved and homologous proteins have been identified in human, mouse, *Xenopus*, *Drosophila* and *C.elegans*. The TAK1 and TAB1 homologues in *C.elegans* (MOM-4 and TAP-1) antagonize Wnt signaling during development[145,220]. Also in mammalian cells and during *Xenopus* development a similar antagonizing effect of TAK1, via NEMO like kinase (NLK) was shown to downregulate Wnt induced β-catenin/ TCF transcriptional activation[92-94]. *Drosophila* dTAK1 plays an important role in innate immunity via regulation of NFκB[228]. Moreover, during Xenopus development TAK1 and TAB1 are implicated in BMP-4 signaling in the central nervous system[203,242]. BMP-4 plays a role in the repression of neural fate of *Xenopus* ectoderm cells[75]. In these studies overexpression of TAK1, resulting in higher levels of activated TAK1 which mimics BMP-4 signaling, enhanced ventral mesoderm formation at the expense of neural differentiation[203,242]. Interestingly, overexpression of TAK1 induced extensive apoptosis and therefore these studies were performed together with overexpression of the anti-apoptotic Bcl-2 protein[203,242].

Besides its role in BMP and Wnt signaling, TAK1 plays a role in several other signaling pathways including the IL-1, IL-18, LPS, and RANK signaling pathways[91,155,216,217,230]. However, besides the relatively well characterized TAB1 protein two other TAB proteins have been identified, named TAB2 and TAB3. In contrast to TAB1 of which its role in TAK1 regulation has been clarified, the role of TAB3 and to a lesser extent TAB2 in TAK1 regulation remains unclear. TAB2 was originally reported as a adapter protein required for interaction of TAK1 and TRAF6 in IL-1R signaling[216]. Moreover, biochemical protein analysis revealed that upon IL-1 stimulation both TAK1 and TAB2 translocate from the plasma membrane to the cytosolic fraction, consistent with the idea that these proteins would first have to interact with the IL-1 receptor in order to transduce signals to other proteins in the cytosol[102,216,217]. Recently, immunofluoresence studies by Beak et al. showed that TAB2 is localized in the nucleus of neuronal cells and translocated to the cytoplasm upon IL-1 stimulation[7]. They proposed that TAB2 in the nucleus fulfilled a role as repressor of NFκB-mediated gene activation[7]. Further evidence that TAB2 might not play a crucial role as an adapter protein in IL-1R signaling, was provided by *Tab2* gene disruption experiments[197]. *Tab2* deficient mice die around E12.5 due to fetal liver degeneration by apoptosis. However, upon IL-1 stimulation *Tab2*-deficient cells respond similarly to wildtype cells in the induction of IL-1 target genes, JNK and NFκB activation, suggesting that TAB2 might not be involved in IL-1R proximal signal

transduction events[197]. Therefore, the exact role of TAB2 in IL-1 signaling remains controversial and to be further elucidated. The last identified TAB protein, TAB3 is implicated in BMP signaling in *Xenopus*. Based on its high homology to TAB2, it was proposed to function as an adapter protein linking TAK1 to the BMP receptor[162].

Transcriptional regulation, Transcription factors and hematopoiesis

Transcription factors play an important role in converting (extracellular) signals into gene expression changes that will result subsequently in the alteration of cellular behavior or properties. As transcription factors fulfill such an important role in mediating changes in cellular behavior and regulating cell fate changes, they have been subject of intense investigation over the years. By means of gene targeting studies several transcription factors, including Runx-1[e] , SCL/tal-1, and GATA-2, have been shown to play an important role in the emergence and/or regulation of HSCs during mouse development[189,192,193,222,223,232] . Besides the transcription factors that are required for the most immature hematopoietic cells, several other transcription factors were shown to play a role in more mature cells within the hematopoietic system. For example, GATA-1 is required for erythroid differentiation[187], Pax5 for B-cell differentiation[170] and Ikaros for lymphoid cell development [221,231]. An overview of several important transcription factors that act within the hematopoietic system is provided in Table 2.

Interestingly, several transcription factors that have been implicated to fulfill a crucial role in mammalian hematopoiesis have also been implicated in *Drosophila* hematopoiesis. The conserved transcription factor of the Rel family, NFκB plays an important role in innate immunity in both mammals and *Drosophila*[204]. Within the *Drosophila* hematopoietic system there are three main cell types. Hemocytes are precursor cells that can differentiate into plasmatocytes or crystal cells. The plasmatocytes resemble monocyte/macrophage like cells and the crystal cells are involved in wound healing and encapsulating foreign invaders[68]. The transcription factor Serpent (GATA homolog) is important for maintenance and proliferation of hemocyte precursors and the transcription factor lozenge (Runx-1 homolog) is important for crystal cell differentiation[68,118].

Within the hematopoietic hierarchy there is a tight regulation of transcription factors that fulfill crucial roles during the differentiation process into mature hematopoietic cells. For several transcription factors it has been shown that down or up-regulation at specific points in the hematopoietic hierarchy is crucial for correct differentiation. For example, for the transcription factor GATA-1 it was shown that both lack of gene expression as well as increased gene expression resulted in inhibition of erythrocyte differentiation, indicating that tight regulation of GATA-1 is prerequisite for normal erythrocyte production[186,187,236]. Also for the transcription factor Runx-1 it was shown that normal Runx-1 function was required for myeloid differentiation and that Runx-1 regulates the gene expression of several myeloid genes, including the transcription factor *Pu.1* [129,173,174]. Besides the expression of these transcription factors required for HSC function, it has been proposed that HSCs (and other stem cells) express at low level a large number of genes and transcription factors that fulfill crucial roles at later stages in the hematopoietic hierarchy. Differentiation of multi-potent cells into a specific mature cell lineage would be

[e] AML-1 has recently be renamed Runx-1 and is also known as PEBP2αB, CBFα2.

accompanied with downregulation of genes required for other mature cell lineages[88]. Within the hematopoietic hierarchy an important commitment step has been proposed to reside at the choice between lymphoid or myeloid differentiation via the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). The two progenitor cell types either express lymphoid specific genes or myeloid specific genes[153]. How these different choices are regulated and how the downregulation of lineage specific genes is regulated is still under investigation.

Table 2. Transcription factors implicated in hematopoietic (stem) cell regulation

Transcription factor	Class	Expression	Phenotype gene targeting studies in mice	Reference
GATA-1	zinc finger	- erythrocytes - mast cells - megakaryocytes - sertoli cells	- embryonic lethal - erythroid differentiation defects	186,187,235
GATA-2	zinc finger	- HSCs/ HPCs - early erythroid cells - mast cells - megakaryocytes - brain, kidney, heart	- embryonic lethal E10-11 - definitive hematopoiesis defects - blood cell differentiation defects	151,184,222, 223
GATA-3	zinc finger	- fetal liver/ thymus - E10-11 dorsal aorta - nervous system - kidney	- embryonic lethal E11.5-12.5 - fetal liver hematopoiesis defects - CNS development defects	32,133,182
Ikaros	zinc finger	- HSCs/ HPCs - lymphoid cells - brain	- HSC/ HPC defects - B and T lymphoid defects	108,165,221, 231
c-myb	Leucine zipper	- HPCs - colonic mucosa - fetal and adult brain	- embryonic lethal E15 - erythroid defects - less CFU-GM progenitors	160,208
NFκB (RelA/p65)	RHD	- ubiquitous	- embryonic lethal E15-16 - fetal liver apoptosis	14,44
NFκB (RelB)	RHD	- lymphoid tissues	- multi-organ inflammation - impaired T cell responses	27,234
NFκB (c-rel)	RHD	- hematopoietic organs - T and B cells	- impaired immune responses	113
NFκB1/ p50	RHD	- ubiquitous	- B cell defects	44,200
PU.1	ETS	- B-cells - myeloid cells - erythroblasts	- embryonic lethal E17-18 - myeloid and lymphoid defects - HSC homing defects	65,66,128,138, 199
Rbtn2/ Lmo2	LIM	- hematopoietic clusters - vascular endothelium - fetal liver/ thymus - embryonic brain	- embryonic lethal E9-10 - erythroid development defects - YS hematopoiesis defects - definitive hematopoiesis defects	133,233,241
Runx-1/ AML-1/ Cbfa2	bHLH	- HSCs/ HPCs - vascular endothelium - nervous system	- embryonic lethal E12.5 - definitive hematopoiesis defects - hemorrhaging CNS	168,174,232
SCL/ tal-1	bHLH	- HSCs /HPCs - erythroid cells - megakaryocytes - mast cells - brain, heart - vascular endothelium	- embryonic lethal E9.5 - YS hematopoiesis defects - YS vascular defects - definitive hematopoiesis defects	12,59,105,189

Runx-1/ AML-1

The *RUNX-1* gene is one of the most frequently mutated genes in human leukemia and was originally identified as a target of the t(8,12) chromosomal translocation that occurs in acute myeloid leukemias[129]. After the original identification, several other chromosomal translocations of the *RUNX-1* gene have been reported, resulting in inappropriate Runx-1 expression or disturbed Runx-1 protein function[129]. Runx-1 belongs to the family of conserved runt homology domain transcription factors. Runx-1 is a helix-loop-helix transcription factor and forms the DNA binding subunit of a heterodimeric CBF (core binding factor) transcription factor, consisting of Runx-1 and CBFβ. Besides Runx-1, two homologous transcription factors have been identified, named Runx-2 and Runx-3 which also dimerize with CBFβ. The CBFβ subunit enhances DNA binding and is involved in transcriptional regulation of the Runx subunit[129]. Runx-1 binds to the DNA sequence TGT/cGGT and regulates several hematopoietic genes including *Gm-csf* and *M-csf receptor, Il-3, Myeloperoxidase, Pu.1, c-Myb, Vav* and *Flk-2* [129,173].

Mice expressing the *lacZ* marker gene under control of the *Runx-1* gene regulatory sequences revealed that Runx-1 expression can be detected between E8 and E10 in primitive erythrocytes in the yolk sac and from E8.5 onwards the endothelial cells of the vitelline artery and dorsal aorta. From E10.5 onwards Runx-1 expression is detected in the liver and in some parts of the nervous system[168]. In the absence of Runx-1 no intra-aortic clusters are found in the dorsal aorta, strongly indicating that Runx-1 is involved in the generation of HSCs during embryogenesis[168]. Further transplantation studies with Runx-1$^+$ and Runx-1$^-$ sorted cells from E11-E12 AGM or fetal liver showed that all LTR-HSC activity is in the Runx$^+$ population[169].

Gene targeting studies in mice in causing disruption of the *Runx-1* gene revealed that *Runx-1* deficient embryos die around E12.5 of gestation due to the absence of fetal liver hematopoiesis[232,174]. Primitive yolk sac hematopoiesis is not affected in *Runx-1* deficient embryos. In addition, *Cbfβ* deficient mice have a similar phenotype as *Runx-1-/-* mice confirming that both subunits of the CBF transcription factor play an important role in hematopoiesis *in vivo*[232]. Furthermore, *Runx-1-/-* ES cells failed to generate myeloid or definitive erythrocyte cells, indicating that Runx-1 indeed plays a crucial role in definitive hematopoiesis[174,232]. Besides the hematopoietic defect, also necrosis and hemorrhaging in the central nervous system was detected in *Runx-1-/-* embryos. Interestingly, when E11 AGM tissue was cultured in an organ culture system, LTR-HSC activity was lost in *Runx-1+/-* AGMs, but not in wild type AGMs, suggesting there is a defect in the AGM microenvironment that normally supports LTR-HSCs[232]. Altogether, these studies indicate an important role for Runx-1 in emergence and regulation of HSCs during development. Most interesting is that lack of Runx-1 expression results in a severe defect in definitive hematopoiesis.

SCL/ tal-1

The *SCL* gene (also known as *tal-1*) encodes a helix-loop-helix transcription factor that binds to the E-box motif CANNTG [15]. *SCL* was originally identified in a case of acute T-cell leukemia with a stem cell phenotype. Since its identification, aberrant expression of the *SCL* gene was found in a large number of cases of T-cell leukemia [15]. Besides its involvement in leukemia, the *SCL/tal-1* gene was found to play an essential role in hematopoiesis. In the mouse embryo SCL protein expression was detected in the

developing blood islands of the yolk sac in both endothelial as well as hematopoietic cells at E8.5. From E9.5 onwards SCL expression can be found in the developing liver and between E10.5 and E14.5 SCL expression further increases, correlating with the increasing hematopoietic activity in the fetal liver[105]. In the human, SCL is expressed in megakaryocytes, erythroblasts, basophilic granulocytes and $CD34^+$ progenitor cells in the peripheral blood and BM, but not in circulating T-cells or B-cells. The expression of *SCL* parallels that of *GATA-1* and both genes are downregulated upon differentiation into mature blood cells[159].

Scl deficient embryos die around E9.5 due to the absence of YS hematopoiesis and abnormalities of vitelline vessels. More detailed analysis revealed that in *Scl* deficient embryos transcripts for *Gata-1, Pu.1* and embryonic *Globin βH1* were absent[193]. Further studies with *Scl*-/- ES cells in chimeric mice and in embryoid body cultures revealed that *Scl*-/- ES cells were unable to contribute to the hematopoietic system or to form hematopoietic colonies[189,192]. This suggests an early block in hematopoietic development, either at the level of specification of the hematopoietic stem cell or at the level of the hematopoietic progenitor cell. Despite its important role during development, SCL expression in the adult mouse might not be crucial for HSC function. Recent studies in which the *Scl* gene was disrupted in adult mice, revealed that bone marrow HSCs lacking a functional *Scl* gene could upon transplantation repopulate the hematopoietic system and give rise to several hematopoietic cell lineages[148].

GATA transcription factors

GATA factors are zinc finger transcription factors that bind to the DNA consensus sequence (T/A)GATA(A/G). The GATA members 1, 2 and 3 are regarded as the hematopoietic GATA transcription factors and GATA members 4, 5 and 6 as non-hematopoietic family members. GATA-1 is essential for erythrocyte differentiation and GATA-3 for T-cell development and hematopoietic precursor regulation. Gene targeting studies for *Gata-1* and *3* revealed that lack of gene expression result in an embryonic lethal phenotype. *Gata-2* was shown to be an important regulator in HSCs by gene targeting studies[32,133,182,186,187,235]. GATA-2 is highly expressed in immature hematopoietic cells and GATA-2 expression is down-regulated upon differentiation into most mature hematopoietic cells[184]. Outside the hematopoietic system GATA-2 expression can be detected in the central nervous system, endothelial cells, liver and kidney (151 and references therein). *Gata-2* deficient mice have an embryonic lethal phenotype and die between E10.5-11.5 of gestation due to severe anemia. More detailed investigations revealed that *Gata-2* deficient mice have reduced numbers of primitive erythrocytes, but that the definitive hematopoiesis was severely disrupted. Studies with *Gata-2* deficient chimeric mice revealed that *Gata-2* -/- ES cells do not contribute to fetal liver hematopoiesis or to hematopoiesis in adult mice. Within a *Rag* -/- background the *Gata-2* -/- ES cells contributed to a small extent to the lymphoid system. Based on these findings is was concluded that GATA-2 expression is required for all hematopoietic lineages[222].

Interestingly, similar to the dosage effects of *Gata-1, Gata-2* overexpression has detrimental effects on hematopoietic progenitor cells. Overexpression of *Gata-2* in the adult bone marrow hematopoietic stem cell compartment (i.e. Sca^+/Lin^- BM cells) resulted in a lower frequency of CFU-S and impaired hematopoietic recovery upon transplantation. However, *Gata-2* overexpression does not result in increased apoptosis [184]. Thus, GATA-2

is an important factor involved in the regulation and possibly emergence, of HSCs during development and also for adult hematopoiesis. The observation that GATA-2 expression can be detected from E9 onwards in the P-Sp/AGM region, the region where the first adult-repopulating HSCs can be detected, strongly suggests that GATA-2 is a regulator of HSCs from the earliest stages onwards.

Rel/NFκB

Nuclear Factor kappaB (NFκB) was first identified as a DNA binding factor regulating the expression of *κ light chains* in mouse B lymphocytes[10]. Numerous studies have shown that members of the NFκB family play an important role in differentiation, apoptosis and proliferation regulation in several hematopoietic cell lineages[10,44,204]. Although the role of NFκB transcription factors has been extensively studied within the (innate) immune system[204], their role in the regulation of HSCs and progenitor cells is less well understood. Members of the Rel/NFκB family are conserved dimeric transcription factors which share a 300 amino acid Rel homology domain (RHD) and can bind to the consensus DNA sequence (κB motif) GGG(A/G)NN(T/C)(T/C)CC[10,44]. In mammals there are 5 known NFκB members, namely RelA/p65, RelB, c-rel, NFκB1 (p105/50) and NFκB2 (p100/p52)[44]. RelA, RelB and c-rel have strong transactivation domains and are generated as mature proteins. In contrast, the family members p50 and p52 are generated from the p105 and p100 proteins and lack strong activation domains. Therefore, these two members are presumed to function as modulators or inhibitors of NFκB-mediated gene regulation. In order to exert their function, NFκB proteins have to homo or heterodimerize and several combinations are possible, although the p65/p50 heterodimer is the most common one[10,44].

As mentioned in the section on IL-1 signaling, NFκB is, in non-stimulating conditions, located in the cytoplasm bound to IκB inhibitor proteins. Upon stimulation, IκB is phosphorylated by IκB kinases (IKK) and targeted for degradation, thereby releasing NFκB and exposing its nuclear localization signal. NFκB is subsequently able to translocate to the nucleus and activate target genes [10,44,204]. For both the IκB and the IκB kinase proteins several homologous proteins have been described, with different affinities for their substrates and different modes of regulation[122,123,137,146]. A large number of NFκB target genes have been identified and these include cytokines, chemokines, adhesion molecules and acute phase proteins and several regulators of apoptosis [9,71,121,240]. Besides IL-1, several other signals can induce NFκB signaling, among which are TNFα, LPS, cellular stress inducing factors and others. Apart from the inducible signals to activate NFκB, also constitutive NFκB gene regulation has been observed. The selectivity of NFκB signaling and gene regulation appears to be regulated through the specific interaction of the NFκB subunits with each other and their target gene promoters, as well as through several levels of regulation within the signaling cascade.

NFκB members play an important role within the innate immune system, in Drosophila as well as in mammals[44,204]. Through gene targeting studies in mice their essential role in the immune system was revealed. *RelB* deficient mice lack dendritic cells and have impaired immune responses as well as multi-organ inflammation[27,234]. Mice lacking c-Rel expression display impaired T and B cell function[113] and mice deficient in *Nfκb1* or *Nfκb2* have B cell defects [8,200]. None of these mutant mice are embryonic lethal, which is in contrast to *RelA* deficient mice which die due to fetal liver apoptosis around E15-16 of gestation[14]. Moreover, *Ikkγ* and *Ikkβ* deficient mice die also due to fetal liver

apoptosis, although slightly earlier during development at E12-13 [123,194]. Together these studies indicate an important role for NFκB signaling during fetal liver development, as mice deficient for either the NFκB regulator *Ikk* or for the NFκB family member *RelA* display fetal liver defects. Functional redundancy between the different NFκB transcription factors, but not between the regulatory IKK proteins might account for the discrepancy between the time of death of the *RelA* deficient embryos (E15-16) and the *Ikk* deficient embryos (E12-13). Furthermore, it was shown that TNFα played a crucial role in the induction of fetal liver apoptosis in *RelA* deficient mice[13].

The HSC genetic program: approaches for novel gene discovery.

Within the hematopoietic system, HSCs are the most immature cells with the ability of extensive self-renewal. The characteristic of self-renewal is an interesting biological property, as most cell types within the adult have limited self-renewal capacity. However, besides HSCs, other types of stem cells with extensive self-renewal capacity have been identified, including neural and mesenchymal stem cells. Most likely, in these stem cells a specific set of genes responsible for self-renewal is expressed, while in other cell types with limited self-renewal properties, these genes are not or differently expressed. Another interesting feature of HSCs is that their descendants differentiate towards lineage-restricted precursors which in turn differentiate towards the mature hematopoietic cells. During this differentiation process the intermediate precursors and mature cells have specific gene expression patterns meeting the requirements of the different functions these cells have to perform. The expression of genes at a transcriptional level correlates with the presence and/or amount of mRNA within a cell. Therefore, comparing the mRNA content between two or more cell populations, cell types or tissues can reveal the difference in gene expression between these cell populations. Moreover, these comparisons can yield more insight into which genes play an important role in certain cell types, such as stem cells, or are involved in processes such as differentiation.

Several studies have applied different techniques to detect the expression of genes within specific populations of cells within the hematopoietic system. These studies compared the gene expression pattern of HSCs and the lineage restricted precursors to gain insight into the regulatory processes along the first steps of the differentiation pathway of the hematopoietic hierarchy[188]. More recently, two studies compared different populations of stem cells to gain insight into the shared characteristics of these cells as compared to differentiated cells[96,191]. Together these studies have provided important insights into the regulation and gene expression pattern of HSCs. As with all studies addressing gene expression differences between two (or more) populations of cells, the purity of the starting population of cells is an important factor in determining the success of the final results. HSCs are a rare population of cells within the bone marrow and also within the embryo. Therefore, to obtain material for these studies, HSCs need to be enriched. The quality and amount of HSCs depends on the enrichment protocol and the purity of the enrichment by FACS sorting. In the cases that the starting population of cells contains a small percentage of HSCs, specific HSC gene expression information might be masked by the gene expression pattern of other cell types within the population. Despite these difficulties, several studies successfully employed these gene expression screenings to unravel parts of the gene expression pattern in HSCs and thereby gain insight into their physiological properties.

One of the first methods developed to monitor changes in gene expression was Differential Display RT-PCR (DD RT-PCR)[125] and a schematic overview of this technique is shown in Figure 4. Since the original description of the technique in 1992 by Liang and Pardee[125] several improvements to the technique have been introduced[158]. Especially the use of longer, more specific primers has improved the reproducibility of the technique[76,124,126]. In addition, methods to screen the large number of DD RT-PCR clones have been introduced [109]. The disadvantage of the DD RT-PCR technique is the large number of false positive clones that are generated and that for a representative overview of differentially expressed genes a large number of primers should be used[124,126]. Most false positive clones are introduced during the excision of the differentially expressed PCR fragments from the polyacrylamide gel. The excised band usually contains, besides the PCR fragment of interest, additional PCR fragments that are not differentially expressed. These latter PCR fragments are the major source of false positive clones and therefore additional screening methods (i.e. virtual Northern blotting and cDNA dot blot expression analysis) have been developed to eliminate these clones. Another drawback that has been suggested is that DD RT-PCR is biased towards identification of highly expressed mRNAs[16]. The major advantage is that DD RT-PCR requires small amounts of starting material (1 µg total RNA is sufficient for a small screening with 8 primers) and that manipulation of the starting material is minimal, thereby reducing the changes of artifacts.

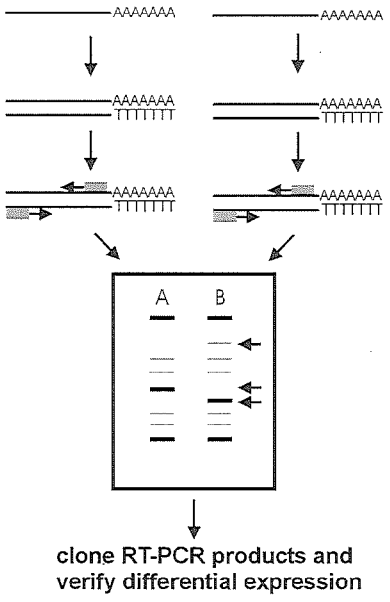

Figure 4. Overview of the Differential Display RT-PCR technique.
RNA from two or more populations of cells or tissues is isolated and cDNA is generated. The radioactive RT-PCR with arbitrary primers is performed under low stringent PCR conditions for 2-3 cycles to allow the primers to bind to non-perfect matched target cDNA. Subsequently high stringent PCR conditions are applied for 30-40 cycles. The RT-PCR mixture is separated on a polyacrylamide gel and exposed to a film. Differential bands can be excised from the gel, purified and cloned for further analysis.

In addition to DD RT-PCR, other methods were developed to identify differentially expressed genes. The suppression subtractive hybridization (SSH) and representational difference analysis (RDA) methods are based on similar principles and have in common that first the common mRNAs between the two starting mRNA population are removed and that only the differentially expressed mRNAs are amplified. These

methods have been suggested to be more sensitive to identify low expressed mRNAs [45,127]. The major disadvantages of these techniques are that a large amount of starting material is required which is manipulated in a large extent (i.e. restriction enzyme digestion and adapter ligation). In addition, changes in expression levels are not easily identified, as common mRNAs are eliminated during the procedure. The major advantages of these techniques are the low number of false positive clones and the high reproducibility. Besides DD RT-PCR and the subtractive screening methods other methods have been developed over the years that are mostly based on the principles of DD RT-PCR and SSH.

The state of the art methods to identify differentially expressed genes are the macro and micro-arrays. These arrays (either a filter or a glass slide) contain a large number of small spots of cDNA (or oligonucleotides) ranging from several hundreds of spots up to several thousand of spots. Hybridization of radioactive or fluorescent labeled cDNA populations allows the analysis of a large number of genes that are differentially expressed between two or more populations of cells. The major advantages of this technique are that in one experiment the expression of a large number of genes can be monitored and the high sensitivity of the technique. However, due to the high sensitivity of this technique, small differences in the isolation and manipulation of the starting material will result in false positive differentially expressed genes. Nonetheless, the micro-array technology allows gene expression profiling of a large number of genes and has proven to be major step forward into the identification of differentially expressed genes.

Scope of this thesis

In this introduction an overview of the current knowledge of HSC development and regulation is provided. Despite the large number of studies on HSCs in the adult, little is known concerning the genetic program for HSC emergence in the mouse embryo. Since HSC function is ultimately based on the genetic program, the aim of this study was to identify (novel) genes involved in hematopoiesis and in particular involved in the emergence/ regulation of hematopoietic stem cells in the mouse AGM region between E10 and E11 of development.

Chapter 2 describes the results of the search for novel genes in the mouse AGM region between E10 and E11 by Differential Display RT-PCR. We found several differentially regulated genes of which some were not previously linked to hematopoiesis and three genes were novel mouse homologues of known human genes. Two of these genes, namely *mTm9sf2* and *mTab2* were further investigated. Chapter 3 describes the expression pattern of TAB2 and related genes. As TAB2 was implicated in IL-1 signaling we investigated the role the role of IL-1 on midgestation hematopoiesis, which is described in Chapter 4. In Chapter 5 we describe our investigations regarding the role of apoptosis and the Bcl-2 protein on hematopoiesis. In the last chapter, the general discussion, we attempt to integrate the findings of these signaling molecules and their possible role in regulating HSCs and hematopoiesis during the earliest stages of development.

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Chapter 2

Identification of two novel genes developmentally regulated in the mouse aorta-gonad-mesonephros region

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Abstract

During mouse development the first adult-repopulating hematopoietic stem cells (HSC) emerge in the aorta-gonad-mesonephros (AGM) region at embryonic day 10.5 prior to their appearance in the yolk sac and fetal liver. Several transcription factors, growth factors and morphogenetic signals are known to play a pivotal role in the onset of adult-type hematopoiesis in the AGM region. However, there are still large gaps in our understanding of the developmental program taking place in the AGM region at the time of HSC emergence. To identify novel developmentally regulated genes that play a role in the emergence of HSCs in the AGM region, we performed Differential Display RT-PCR. We detected several differentially expressed genes in the AGM region between E10 and E11, including *β-catenin* and two novel mouse genes highly homologous to human *TM9SF2* and *TAB2*. Other components of the Wnt/β-catenin signaling pathway were also detected in the AGM and fetal liver, suggesting a role for Wnt signaling in AGM hematopoiesis. We characterized the expression pattern of the novel mouse genes, *Tm9sf2* and *Tab2*, in the embryo and adult by Northern blotting and RT-PCR. More importantly, the specific expression of mTAB2 protein in the endothelium of the E11 dorsal aorta (the site known to harbor HSCs) suggests a role for mTAB2 in HSC emergence and/ or regulation. These studies identifying novel differentially expressed genes in the midgestation AGM region should yield further insights into the development of this tissue and into the emergence and regulation of HSCs.

Introduction

The stem cells that form the basis of the hematopoietic hierarchy in the adult bone marrow are characterized by their ability to self-renew as well as undergo extensive differentiation and proliferation. Hematopoietic stem cells (HSC) are functionally identified by their ability to reconstitute the entire blood system when injected into HSC-ablated adult animals (reviewed in 24,49). During mouse development, such adult-repopulating HSCs first appear during midgestation (embryonic day 10.5; E10.5) within the embryo proper in a region containing the developing vascular and urogenital tissues. This mesodermally-derived region is referred to as the dorsal aorta-gonad-mesonephros (AGM) region [9,29,32]. More detailed studies show that HSCs are localized within the dorsal aorta (and also the vitelline and umbilical arteries) [10,11]. The HSCs autonomously generated in the aorta during midgestation are as potent as those residing in the adult bone marrow (BM) [32]. Slightly later at E11, HSCs are found also within the yolk sac (YS) and the fetal liver [32]. Spatial and temporal mapping of HSCs suggest a lineage relationship that is consistent with the general notion that following their generation, HSCs migrate and colonize the liver during fetal stages and subsequently the bone marrow where they reside throughout adult stages of life[23,29]. Thus, since the *de novo* production of HSCs is thought to take place only in a limited time within the embryo, the AGM region is of particular interest for the study of the signals and genes involved in the generation, proliferation and regulation of earliest HSCs. An increased understanding of these signals under normal physiological circumstances will result in a better understanding of deregulated hematopoiesis, such as in leukemia.

Research has focused on the influence of the microenvironment, the role of known morphogenetic signals, growth factor receptors and transcription factors leading to the induction of HSCs in a specific temporal and spatial manner within the embryo. To date, some of the morphogenetic signals required for the correct spatial and temporal formation

of most tissues and organs during development, i.e. members of the Hedgehog, TGFβ and Wnt families (reviewed in 60,8,17,27,28,14) are being examined for their role(s) in the induction of HSCs. For example, hedgehog family members IHH and SHH respectively induce hematopoietic/vascular cell fate during early mouse development [15] and the proliferation of human hematopoietic progenitors [5]. In addition, BMP-4 (TGFβ family member) is implicated as mediator of these effects and both BMP-4 and TGFβ1 (another TGFβ family member) play a role in the development of both hematopoietic and vascular systems in the mouse embryo [7,13,48,59]. The expression pattern of BMP-4 on the ventral side of the dorsal aorta in the human AGM further supports a role for BMP-4 in HSC generation [26]. Also, Wnt signaling has been shown to affect hematopoiesis. Members of the Wnt family induce the proliferation of mammalian hematopoietic progenitor cells[1,55] and Wnt11 is required for the formation of all hematopoietic lineages in avian embryos [6]. Taken together, these known developmental modulators appear to play important instructive roles during hematopoietic and vascular development.

Other studies have addressed the role of known growth factors and transcription factors in the induction and regulation of HSC during development. Some pivotal growth factor receptors involved in these processes are c-kit and Flk-1 [41,44,45] and some pivotal transcription factors are Aml-1, Gata-2 and Scl/tal-1 [33,36,39,53]. While these genes are required for fetal liver/adult hematopoiesis, *Scl/tal-1* and *Flk-1* also affect the development of the vasculature [39,45,64] suggesting the existence of a common precursor for both lineages, the hemangioblast. Such genetic evidence has supported the notion that hemangioblast-type cells are the direct precursors to AGM HSCs in the midgestation embryo [19].

Despite these studies, which have addressed the role of known genes in the emergence and regulation of HSCs during development, there are many gaps in our understanding of the genetic program of HSCs and the inductive microenvironment within the AGM. In order to further increase our understanding of the properties and genes involved in the emergence and regulation of HSCs in the AGM, we set out to identify novel genes that are differentially expressed in the AGM at the time of HSC induction. Several gene screening and cloning techniques (i.e. Differential Display (DD) RT-PCR, SAGE and micro-array analysis etc.) have been developed and have successfully identified panels of differentially expressed genes between two (or more) distinct hematopoietic cell populations [35,58]. Using the non-biased DD RT-PCR technique, we performed screenings on AGM tissues derived prior to (at early E10) and subsequent to (at E11) the emergence of HSCs to identify new genes. We report here the identification several genes that are up- or down-regulated in the AGM at the time of HSC induction, including *β-catenin*, a member of the Wnt signaling pathway, and two novel mouse genes homologous to a human transmembrane protein *TM9SF2* and human *TAB2*, a member of the IL-1 signaling cascade. We provide evidence that suggests a role for *β-catenin* and other Wnt signaling components in the AGM. Characterization of the two novel mouse genes, *Tm9sf2* and *Tab2*, similarly suggest a role for these genes in hematopoiesis. Most interestingly, the localized expression of mTAB2 within the endothelium of the E11 dorsal aorta, suggests an important role for *Tab2* in the emergence and/or regulation of HSCs during development.

Material and Methods

Embryo generation and cell lines

To generate embryos, matings were set up between C57BL/6 or (CBA x C57BL/10)F1 males and females. The day of vaginal plug discovery is E0. Mice were killed by cervical dislocation and embryos were collected in PBS/ 10% FCS. AGM, aorta-mesenchyme (AM) and gonad-mesonephros (UGR) tissues were dissected as described previously [11,32]. 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. Cell lines were cultured as described previously [54,57].

RNA isolation, cDNA synthesis and Northern blotting

Total RNA from embryonic and adult mouse tissues was isolated with Ultraspec (Biotecx) and Trizol (Gibco/ Life-Technologies) respectively, according to manufacturer's instructions. RNA was dissolved in dH_2O, integrity verified on 1% agarose/TBE gels and concentration determined by OD_{260} measurement. Prior to cDNA synthesis RNA was 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 (Gibco/ Life Technologies). cDNA was diluted to the desired concentration with dH_2O.

For Northern blots 10-25 µg total RNA was separated on formaldehyde gels and blotted with 20x SSC in an upward capillary manner to Genescreen membranes (NEN technologies). Blots were probed with α-^{32}P-dATP labeled DNA fragments that were generated with a nick translation kit (Amersham Pharmacia Biotech).

Differential Display RT-PCR

Differential Display RT-PCR was performed essentially as described [20] with some minor modifications. Briefly, total RNA was isolated from E10 (30-35 somite pairs) AGMs and E11 (40-50 somite pairs) AGMs, AMs and UGRs. 1 µg of total RNA was reverse transcribed with Superscript II and purified with PCR spin columns (Qiagen) and eluted in 100 µl TE. DD RT-PCR was carried out with 2 µl of cDNA, 200 ng of degenerate primer, 20µM dNTPs, 1 µCi α-^{33}P-dATP (Amersham), with 4U of AmpliTaq and provided buffer (PerkinElmer) in a total volume of 20 µl. PCR cycles were as follows: 3 min 95°C, 2x (1 min 95°C, 5 min 37°C, 5 min 37-72°C, 5 min 72°C), 30-35x (1 min 95°C, 2 min 55°C, 5 min 72°C), 10 min 72°C. In total 8 arbitrary primers were used with a length of 19-21 bp and approximately 50% GC content. Primers were tested for their ability to produce a clear banding pattern in the DD RT-PCR procedure. Primer sequences are available upon request.

5 µl of the PCR sample was mixed with an equal volume of 96% formamide loading buffer, boiled for 2 min and was separated on a 6% acrylamide/ Urea gel. The gel was dried and exposed to Biomax MR Films (Kodak). Differential bands were excised and DNA was eluted in TE and amplified by PCR using the same degenerate primer as used for identifying the differential band. The PCR product was cloned into PGEM-T easy vector (Promega) and clones were screened for false positives as described [20]. Potential differential clones were sequenced and sequences were run through NCBI non-redundant gene and EST databases to identify the corresponding gene or EST. A small number of clones was verified for differential expression by cDNA dot blotting, RT-PCR or Northern blotting.

cDNA dot blots

RNA and cDNA from several embryonic and adult mouse tissues, as well as hematopoietic cell lines was prepared as described. An equivalent of 1 µg, 200ng and 50ng of each sample was dot blotted with a vacuum dot blot apparatus onto Genescreen membrane (NEN technologies). Dot blots were probed with ^{32}P-dATP labeled DNA fragments excised from differential display clones that were generated with Klenow enzyme (Roche). Results were further processed with a PhosphorImager (Molecular Dynamics) and ImageQuant software.

RT-PCR analysis

RNA and cDNA from several embryonic mouse tissues was prepared as described. RT-PCR was performed in a 50µl volume with 100ng of each primer (listed in Table 1), 0.2mM dNTPs, AmpliTag and provided buffer (PerkinElmer) together with a 40ng RNA equivalent of cDNA. RT-PCRs were performed at an optimized Tm (55°C-62°C) for each primer set. Cycling conditions as follows 5min 92°C, 30-40x (45sec 92°C, 45sec Tm, 1-2min 72°C), 7min 72°C.

Table 1. Primers used for RT-PCR analysis

Primer name	Sequence (5'-3')	Product size	Acc. number
mTAB2-ORF forw	ATACGAATGGCCCAAGGAAGC	2085	BC004813
mTAB2-ORF rev	AGAAATGCCGAGGCATCTCAC		
mTM9SF2 forw	CTGGTTGCCATCTTGGCCCTCTG	854	NM_080556
mTM9SF2 rev	GCTATCGTATTTACAAATGCAC		
β-catenin forw	ATCCAAAGAGTAGCTGCAGGGG	619	NM_007614
β-catenin rev	CCTAAAACCATTCCCACCCTACC		
Dvl2 forw	GAGACGAAGGTGATTTACCAT	308	NM_007888
Dvl2 rev	TGGAGGCGGAGGAACCAGTTC		
Cu/ Zn SOD forw	CATCCACTTCGAGCAGAAGG	442	X06683
Cu/ Zn SOD rev	AGTCTGAGACTTCAGACCAC		
BUB1B forw	GTTTGGTACCAGTATATAAAC	941	NM_009773
BUB1B rev	AATACACAGTGAAAGGAGA		
β-actin forw	CCTGAACCCTAAGGCCAACCG	398	X03672
β-actin rev	GCTCATAGCTCTTCTCCAGGG		
Dvl forw	GAGACCAARATCATCTACCAC	374	U10115
Dvl1 rev	GCTACTGGCAACATTTGGTTG		
Dvl3 rev	TGCGTGCTCCGGGCCATC	458	U41285
TCF1 forw	GCYATYAACCARATYCTSGG	566	X61385
TCF1 rev	TGGGAGCTGGTCATGTTGCC		
LEF rev	TCGCTGCCTTGGCTTTGCAC	270	NM_010703
TCF3 rev	GAGACAGCCTGCATAGAACC	673	AJ223069
TCF4 rev	AAGCTTGGATCTGAAGAGGG	409	AJ223070
Wnt5A forw	GACAGTATACAACCTGGCAG	379	M89798
Wnt5A rev	AGCGTTCGGTCTGCACTGTC		
Wnt3 forw	GAAGGATCCTGGGCATGACAG	447	NM_009522
Wnt3 rev	GTGTGCAGCCTCTCTTCCTC		

Western blot and Immunohistochemistry

A confluent 9 cm dish of 3T3 cells and $5x10^7$ MPRO cells were lysed in RIPA buffer and further processed as recommended by the manufacturer of the TAB2 antibody. $5x10^7$- 10^8 Jurkat and Ros17 cells were lysed in Laemlli sample buffer. Protein samples were separated on a 10% SDS polyacrylamide gel according to standard procedures and blotted against Immobilon-P (Millipore) PVDF membrane. The blot was incubated with anti-TAB2 antibody and subsequently visualized with HRP-conjugated anti-goat antibody and Enhanced Chemiluminesence (ECL, Amersham).

Embryos were snap-frozen into TissueTek O.C.T. compound (Sakura). Sections of 6-8 µM were generated with a cryostat apparatus and placed onto Superfrost glass slides (Menzel-Glaser). Sections were fixed with 2% paraformaldehyde/ PBS and incubated with anti-TAB2 antibody (sc-11851 Santa Cruz). Staining was visualized with the ABC staining kit (Santa Cruz) and sections were dehydrated and embedded in Entallan (Merck).

Results

Isolation and identification of genes differentially expressed in the AGM region between E10 and E11

To increase our understanding of the genetic program involved in the induction and regulation of HSCs in the midgestation mouse AGM, DD RT-PCR was performed (Figure 1A). Three independent DD RT-PCR screening rounds were performed on AGM material obtained prior to (early E10) and subsequent to (E11) the induction of HSCs. DD RT-PCR was performed as described previously with 8 arbitrary primers[20].

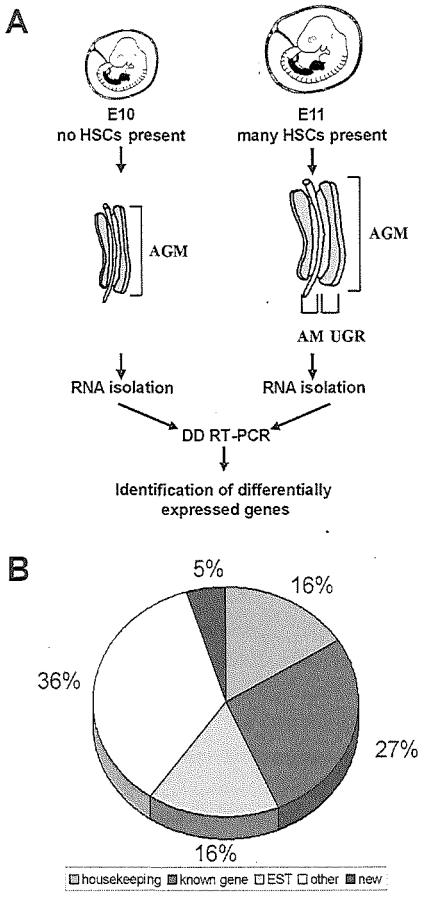

Figure 1. DD RT-PCR Strategy.
Overview of the strategy and results identifying differentially expressed genes in the E10 and E11 AGM. (A) AGM material from mouse embryos at E10 and E11 was collected for RNA isolation. Generated cDNA from these tissues was used to perform DD RT-PCR to detect differentially expressed genes. (B) Summary of the results from three DD RT-PCR screening rounds. In total 62 clones derived from the DD RT-PCR were sequenced and run through the NCBI EST and non-redundant nucleotide databases. Clones were classified either as housekeeping or known genes, new mouse genes, ESTs or other sequences based on the sequence retrieved from the NCBI nucleotide databases with the highest homology to the sequence of the subsequent clone. In total 43% of the clones corresponded to known genes of which 16% were housekeeping genes and 27% other known genes. 16% of the clones corresponded to ESTs and 36 % of the clones were classified as other sequences and corresponded to either ribosomal RNA, DNA fragments or had no homologous DNA sequence present in the NCBI nucleotide databases. In 5% of the clones (3 clones) we found only a human homologous gene and hence, these clones are referred to as novel mouse genes

For the first screening, RNA was extracted from complete E10 and E11 AGMs (containing the aorta, mesenchyme and urogenital ridges). This screening yielded 15 differential (up- and down-regulated) bands that were excised from the sequencing gel and cloned. DNA from each excised band was cloned into PGEM-Teasy vector and 8 resulting colonies were analyzed in the false positive screening as previously described[20]. Subsequently, the nucleotide sequence of 1-2 clones from the corresponding differential band was determined and run through the NCBI nucleotide non-redundant and EST

databases. In general, the inserts of the clones were approximately 200-600 bp long and represented the 3' mRNA sequence. This first screening yielded clone E1-2 with a novel mouse gene sequence, highly homologous to the human transmembrane protein, *TM9SF2*. Other clones (see Table 2) were found to have sequence homology to several different ESTs, and the mouse *Bub1b*, *Fibronectin* and *Dvl2* genes.

To isolate genes more specifically up- or down-regulated during the onset of HSC induction in the aorta of the AGM, a second screening was performed on RNA isolated from subdissected AM tissue derived from E10 and E11 AGMs (Figure 1A). DD RT-PCR screening was performed, yielding 15 differential bands. Clones corresponding to *Globin epsilon*, *Cu/Zn superoxide dismutase (Cu/Zn SOD)*, *Trap150*, *Sag* and a large number of housekeeping genes were identified. In addition, a novel mouse gene was identified and found to be homologous to human *lipoprotein binding protein* (Table 2).

In the third screening, RNA extracted from complete E10 AGM and subdissected E11 AM and UGR material was used for DD RT-PCR. The use of UGR material allowed for the discrimination and exclusion of bands related to this subregion of the AGM. The screening yielded 12 differential bands. The sequence of clone S1-7 revealed that it was a novel mouse gene highly homologous to human *TAB2*. Clones corresponding to *β-catenin* and some ESTs were found, along with a large number of clones with no homology to sequences in the NCBI non-redundant and EST databases (Table 2).

Table 2. Summary of a limited number of sequenced clones derived from DD RT-PCR screening

Clone (screening)	Expression profile		Accession number	Gene homology
	DD RT-PCR*	Dot blot/ RT-PCR*		
E1-2 (1)	↓	↑↑	NM004800	human *TM9SF2* homolog
H1-3 (1)	↓	↓	BC024644	mouse *Enolase/ myc binding protein*
G2-5 (1)	↓	similar	AF107296	mouse *Bub1b*
G4-1 (1)	↓	similar	NM007888	mouse *Dvl2*
K1-5 (2)	↑	similar	X06683	mouse *Cu/ Zn superoxide dismutase*
K1-6 (2)	↑	↑	M26897	mouse *Globin epsilon*
M2-2 (2)	↓	↑	NM005119	mouse *Trap150*
M2-7 (2)	↓	ND	U90725	human *lipoprotein binding protein*
P1-1 (2)	↑	ND	AF092877	mouse *Sag*
S1-2 (3)	↑	↑	NM019868	RNA binding protein
S1-7 (3)	↑	↑↑	AF241230	human *TAB2*
S2-4 (3)	↑	↑↑	NM007614	mouse *β-catenin*
S2-6 (3)	↑	similar	BG071065	EST

* transcripts that are expressed at higher levels in E11 aorta/ AGM tissue in comparison to E10 AGM are referred to as upregulated (indicated by ↑) and transcripts that are expressed at lower levels in E11 aorta or AGM tissue in comparison to E10 AGM are referred to as downregulated (indicated by ↓). ND is non determined.

In total, 62 cloned sequences derived from the DD RT-PCR were run against NCBI non-redundant and EST nucleotide databases. Based on the highest homology with the sequence retrieved from NCBI nucleotide databases, the corresponding DD RT-PCR clone was classified either as a known or housekeeping gene, EST, new mouse gene or other sequence. The combined results of the three DD RT-PCR screening rounds are summarized in Figure 1B

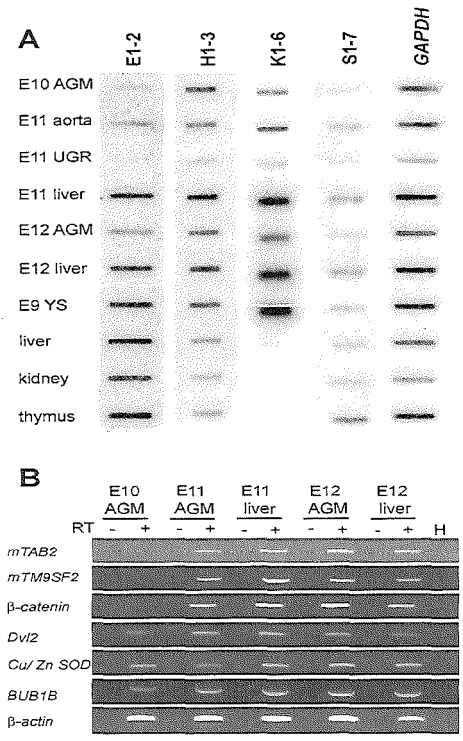

Figure 2. Expression patterns of DD RT-PCR clones and other related genes.
Dot blot screening or RT-PCR analysis was performed with RNA derived from embryo and adult tissues to determine gene expression patterns of some DD RT-PCR clones and related genes. (A) For dot blot analysis RNA isolated from a panel of tissues was used for cDNA synthesis. cDNA (equivalent to 1μg RNA) was dot blotted on a nitrocellulose membrane which was probed with ^{32}P-labelled DNA fragments derived from DD RT-PCR clones to verify differential expression. As a control the blot was stripped and reprobed with GAPDH to check for equal amounts of dot blotted cDNA. The corresponding homologous genes of the indicated clones are listed in Table 1.(B) For each primer set, optimal PCR conditions and annealing temperature was determined and PCR was performed with 30-40 cycles (H is the water control). Three of the genes, Tab2, mTm9sf2 and β-catenin are up-regulated between E10 and E11 in the AGM. The other genes that did not show a differential expression pattern represent false positive DD RT-PCR clones.

To verify that the sequenced clones derived from the DD RT-PCR are differentially expressed in embryonic and adult hematopoietic tissues, we performed dot blot screening (Figure 2A). In addition, RT-PCR analysis was used in some cases as a more specific means to verify differential expression (Figure 2B). We found by cDNA dot blot analysis that *Globin epsilon* (K1-6), *Enolase* (H1-3), *Trap150* (not shown) and the two novel mouse genes *Tm9sf2* (E1-2) and *Tab2* (S1-7) are differentially expressed in the AGM between E10 and E11. *Globin epsilon* (clone K1-6) is up-regulated between E10 and E11 in the AGM, with highest expression in the E9 YS and E11 and E12 fetal liver. The high expression levels in these tissues correlates with the presence of erythroid cells in these sites. As expected, no *Globin epsilon* expression was found in any of the adult tissues examined. *Enolase* (H1-3) was down-regulated between E10 and E11 in the AGM, but high levels of expression could be detected in E9 YS and fetal liver. Both novel mouse genes *Tm9sf2* (E1-2) and *Tab2* (S1-7) were up-regulated between E10 and E11 in the AGM and both were also expressed in the fetal liver. *Tm9sf2* was expressed at high levels in adult tissues and *mTab2* expression could also be detected in adult tissues, with highest

expression in the thymus. RT-PCR analysis further confirmed the differential expression of the two novel mouse genes in the AGM between E10 and E11. Also, up-regulated *β-catenin* expression was verified between E10 and E11 AGM. In contrast, several clones including *Dvl2, Bub1b* and *Cu/Zn SOD* were found not to be differentially expressed in our cDNA dot blot analysis (not shown) or by RT-PCR (Figure 2B) and thus, these clones represent false positives. From these data we conclude that three DD RT-PCR clones representing two novel mouse genes *Tm9sf2* and *Tab2*, and *β-catenin* are up-regulated in the AGM between E10 and E11. We further investigated these three clones.

Several Wnt signaling components are up-regulated in the AGM region between E10 and E11

Wnt genes encode a large family of secreted proteins that act as intercellular signaling molecules in several developmental processes (as reviewed in 8,60), including the regulation of hematopoietic cells [1,38,55]. Since *β-catenin* and *Dvl2* were identified in our DD RT-PCR screening, we next examined the expression of several Wnt signalling components by RT-PCR (Figure 3). This analysis verified the upregulation of *β-catenin* in the AGM after E10, with high level expression in E11 and E12 AGM and E11 liver. While *Dvl2* expression is constant, expression of *Dvl1* and *Dvl3* is up-regulated between E10 and E11 in the AGM. Compared to *Dvl2* and *3,* the expression of *Dvl1* is low in all tissues examined. In addition, the transcription factor genes *Lef, Tcf1* and *Tcf3* (not shown) are expressed, but *Tcf3* and *Lef* are expressed at low levels in the fetal liver and AGM. *Wnt5a* is up-regulated between E10 and E11 in the AGM and is found also in E11 and E12 liver. Although at very low levels, *Wnt3* expression was detected in E10 AGM, E12 AGM and liver (data not shown). Thus, several components of the Wnt signaling pathway are transcribed in both the AGM and fetal liver, with some of the Wnt signaling components up-regulated in the AGM at the important E10 and E11 developmental time when HSCs are being generated.

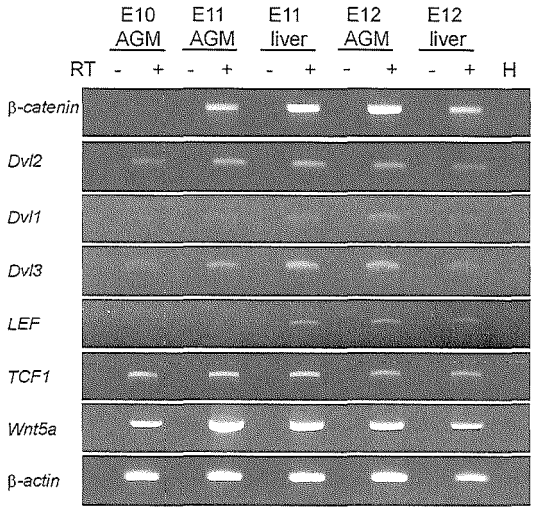

Figure 3. RT-PCR analysis reveals expression of several components of the Wnt signaling pathway in the AGM region and fetal liver.
RT-PCR analysis was performed for several components of the Wnt signaling pathway. Primers are listed in Table 1 and specific PCR conditions and the number of PCR cycles (30-40) were optimized for each primer set. (H is the water control) Several components of the Wnt signaling pathway can be detected in the AGM and fetal liver between E10 and E11. Besides the differential expression of β-catenin between E10 and E11 in the AGM, we could also detect upregulation of Dvl1 and 3, and Wnt5a in the AGM between E10 and E11.

A highly conserved 9 transmembrane protein encoding gene is differentially expressed in the AGM region

The isolation of the differentially expressed E1-2 clone and its identification as a novel mouse gene encoding a transmembrane protein highly homologous to human *TM9SF2* (also known as human p76 or emp70) prompted our further investigations into its sequence and structure. The human TM9SF2 protein contains 9 transmembrane regions but no other obvious protein domains. It is localized in endosomes and is proposed to function as a channel or small molecule transporter [43]. When the human *TM9SF2* sequence was blast searched against the NCBI mouse EST database, several ESTs spanning the entire open reading frame (ORF) of human *TM9SF2* were found (data not shown), strongly indicating the existence of a mouse *Tm9sf2* homologue. Soon after our identification of clone E1-2, a full-length clone of mouse *Tm9sf2* was submitted to the NCBI sequence database by the IMAGE consortium (accession number BC003862).

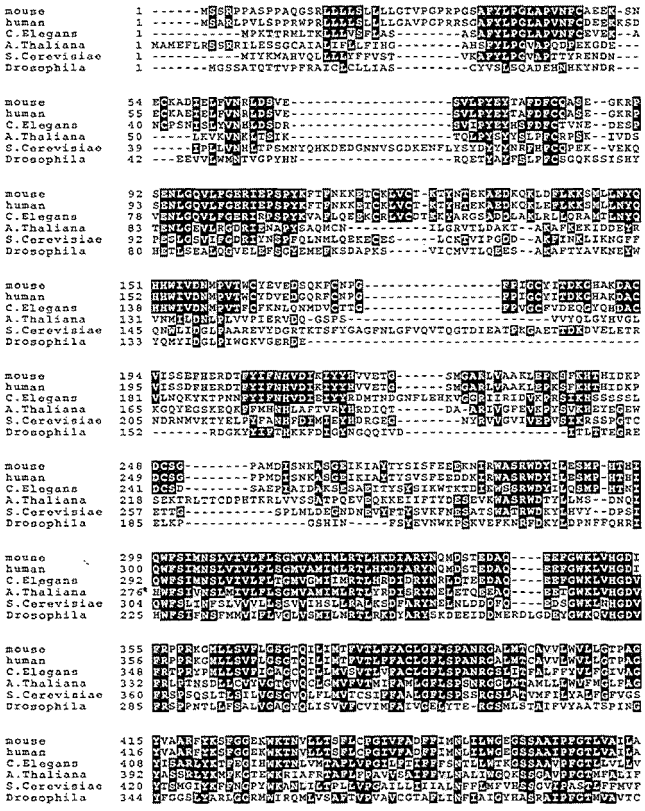

Figure 4. High homology between different species indicates conservation of the TM9SF2 protein. *Alignment of mouse TM9SF2 protein sequence (NM_080556) with homologous proteins from several species including human (Q99805), C.elegans (NP_509429), Drosophila (AAF50762), S.cerevisiae (S64915) and A. thaliana (T50793). Accession numbers of the protein sequences are indicated by the numbers in the brackets and the alignment of the proteins was performed with ClustalW software. Conserved amino acid residues are indicated by the black boxes and similar amino acid residues are indicated by gray boxes.*

We compared the mouse protein to that of the human and found 95% homology (Figure 4). Alignment of the mTM9SF2 protein sequence with similar proteins from *Drosophila, C.elegans, S.cerevisiae, A.thaliana* also shows high homology, suggesting conservation of this gene and its function throughout evolution (Figure 4). To gain more insight into the genomic structure of *Tm9sf2*, the mRNA sequence available from NCBI nucleotide database (BC003862) was blast against the Celera mouse genome database.

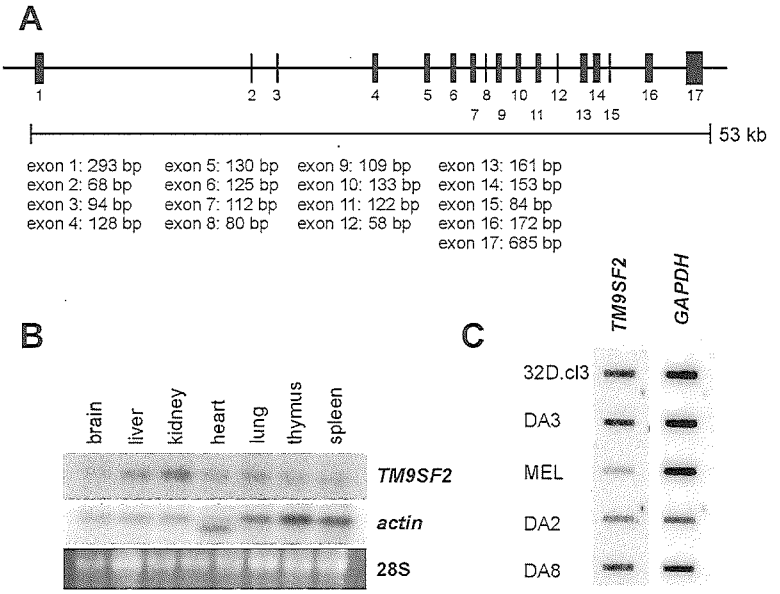

Figure 5. Structure and expression pattern of the mouse TM9SF2 gene.
(A) Schematic representation of the mouse Tm9sf2 gene. The mRNA sequence of Tm9sf2 (BC003862) was blast against the Celera mouse genome database. The mouse Tm9sf2 gene contains 17 exons spanning approximately 53 kb and is located at mouse chromosome 14. The intron-exon boundaries are based on the consensus splicing sequence and therefore, the actual exon lengths may differ by 1-2 bp from the predicted exon length (B) Northern blot probed with Tm9sf2 shows specific expression in all adult mouse tissues examined with highest expression in liver and kidney. The probe hybridizes to a fragment of approximately 3-3.5 kb. (C) cDNA dot blot analysis shows Tm9sf2 expression in several hematopoietic cell lines, with highest expression in myeloid (32D.cl3, DA3) and B-lymphoid (DA8) cell lines.

Alignment of the 2746 bp mRNA sequence of *Tm9sf2* with mouse genomic sequences revealed 17 exons containing the complete ORF, spanning approximately 53 kb of the genome, with the ATG start codon in the first exon (Figure 5A). Alignment of the 2.8 kb *hTM9SF2* transcript to human genomic sequences revealed that the structure of the *hTM9SF2* and *mTm9sf2* genes is similar, but *hTM9SF2* spans approximately 61 kb of the genome. *mTm9sf2* is located on chromosome 14 and *hTM9SF2* is located on chromosome 13q11-34. However, since Northern blot analyses for *mTm9sf2* (Figure 5B) and *hTM9SF2* (Schimoller et al.) indicate that both transcripts are slightly longer than the mRNA sequences in the NCBI database, it is possible that both genes contain additional exons with 5'UTR or 3'UTR mRNA sequences.

To further characterize the expression of *Tm9sf2* in the adult mouse, we hybridized a Northern blot containing RNA from several adult mouse tissues with a probe derived from clone E1-2. The probe hybridized to a fragment of approximately 3-3.5 kb and *Tm9sf2* expression was detected in all tissues examined, with highest levels in kidney and liver (Figure 5B). This corresponds in part to the expression pattern in human tissues, where the highest expression levels are detected in heart, brain, muscle, kidney and pancreas [43]. To investigate *Tm9sf2* expression in hematopoietic cells, we hybridized a dot blot containing cDNA from several hematopoietic cell lines with the E1.2 probe. *Tm9sf2* expression was

detected in all hematopoietic cell lines examined, with highest levels in myeloid (32D.cl3, DA3) and B-lymphoid (DA8) and lower levels in erythroid (MEL) and T-lymphoid (DA2) and cell lines (Figure 5C). Thus, the differential expression of *Tm9sf2* in the E10/E11 AGM and expression in hematopoietic tissues and cell lines suggest that is an interesting gene for further studies in hematopoietic cell development.

Differential expression of *mTAB2*: a component of the IL-1 signaling pathway

Previously, human TAB2 has been shown to be part of the IL-1 and RANK signaling cascade as a binding partner of TAK1 (TGFβ activated kinase 1) [30,51]. Further studies showed that the TAK1/ TAB1/TAB2 protein complex is involved in NFκB and JNK activation upon IL-1 stimulation of cells [30,37,51]. Both IL-1 and RANK function within the hematopoietic system and NFκB is an important hematopoietic transcription factor [12,14,21,52,61]. Our identification of clone S1-7 in AGM DD RT-PCR screening and the verified up-regulated expression between E10 and E11 of the mouse homologue to human *TAB2* prompted further studies.

To investigate the gene structure of mouse *Tab2* and to compare it to human *TAB2*, we retrieved both genomic sequences from the Celera genome database. Alignment of both mRNAs to the genomic sequences revealed 6 exons, with similar intron-exon boundaries containing the complete ORFs of the two genes. The mouse *Tab2* gene spans approximately 20kb and the ATG start site is located in the second exon (Figure 6A). *Tab2* is located on mouse chromosome 10 in a region homologous to human chromosome 6q25, where human *TAB2* is located. The high level of conservation of the genomic structure of *TAB2* between mouse and human suggests that these genes are regulated in a similar manner. Furthermore, the high homology in nucleotide sequence of the ORF and also the high homology of 94 % at the protein level (Figure 6B) suggest conservation of function between mouse and human. Protein motif searches in bioinformatics programs, including Prosite (http//us.expasy.org/prosite), SBASE (http//www3.icgeb.trieste.it/~sbasesrv) and CD-search (NCBI) revealed a coiled coil domain (aa. 529-619). No other domains with high homology were found based on the primary amino acid sequence. However, a search for conserved domains in the CD-database of NCBI indicated that there was homology to a CUE (coupling of ubiquitin conjugation to ER degradation) domain at the N-terminus and a Zinc finger domain at the C-terminus of TAB2. The CUE domain is suggested to play a role in ubiquitination of TAB2.

We verified the up-regulated expression of *Tab2* in the AGM between E10 and E11 by Northern blotting (Figure 6C). Full-length transcripts approximately 3.5-4 kb were also detected in E11 liver. When a Northern blot with adult tissue RNA was probed with the DNA fragment of clone S1-7 (Figure 6D) *Tab2* transcripts were detected in all tissues examined: with highest expression levels in kidney, thymus, spleen and BM and lower levels in liver and brain. Additionally, by cDNA dot blot analysis (Figure 6E), high level expression of *Tab2* was observed in two myeloid cell lines (32D.cl3 and DA3) and low level expression in B and T lymphoid (DA8 and DA2) and erythroid (MEL) cell lines. Thus, by expression pattern and putative function based on homology with the human protein, mTAB2 represents an interesting molecule for further study in hematopoietic development. A full-length IMAGE clone of *Tab2* was deposited into the sequence database of the NCBI by the IMAGE consortium (accession number: BC004813), after our identification of clone S1-7.

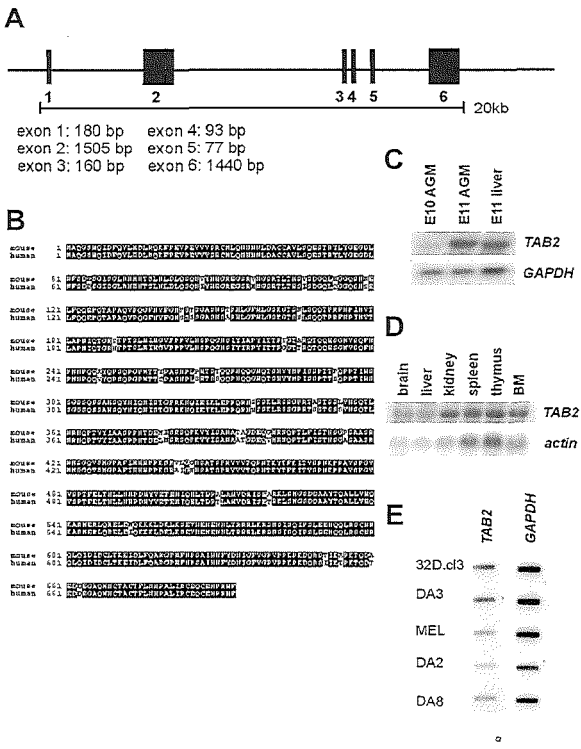

Figure 6. Structure and expression of the mouse Tab2 gene.
(A) Schematic representation of the mouse Tab2 gene. The mRNA sequence of mouse Tab2 (BC004813) was blast against the Celera mouse genome database The Tab2 gene contains 6 exons spanning 20 kb and is located at mouse chromosome 10. (B) Alignment of mouse and human TAB2 protein shows 94% homology indicating conservation of the gene. (C) Northern blot showing differential expression of Tab2 in the AGM between E10 and E11. (D) Northern blot revealing high levels of Tab2 expression in the adult mouse kidney, thymus, spleen and BM. (E) cDNA dot blot analysis shows high expression of Tab2 in two myeloid cell lines (32D.cl3 and DA3) and lower expression in B and T lymphoid (DA8 and DA2) and erythroid (MEL) cell lines

mTAB2 is expressed in the endothelium of the dorsal aorta at E11 and E12

To investigate the specific expression pattern of mTAB2 in the AGM, immunohistochemistry was performed. First we tested a commercial antibody raised against human TAB2 on a Western blot for its ability to recognize the highly homologous mTAB2 protein. Protein extracts from two mouse (fibroblast and myeloid) and two human (B and T lymphoid) cell lines were used for Western blotting. In all extracts a single band of the predicted size of 77 kD was detected (Figure 7A).

Cryosections of E10-E12 embryos at the level of the AGM were prepared for TAB2 immunostaining. In both E11 and E12 trunkal sections we could detect high levels of mTAB2 expression in cells within and lining the neural tube, including the neuroepithelial cells; the dorsal root ganglia and the marginal layer (data not shown). Interestingly, mTAB2 expression was also detected in the endothelium of the E11 (not shown) and E12 dorsal aorta and some underlying mesenchymal cells (Figure 7B). Consistent with the low to undetectable levels of *mTAB2* RNA in the E10 AGM, no mTAB2 expression was found in E10 dorsal aorta endothelium and neural tube. Thus, mTAB2 is expressed in the endothelium of the E11 AGM at the time when HSCs are first being generated, suggesting a role for mTAB2 in the induction or regulation of HSCs

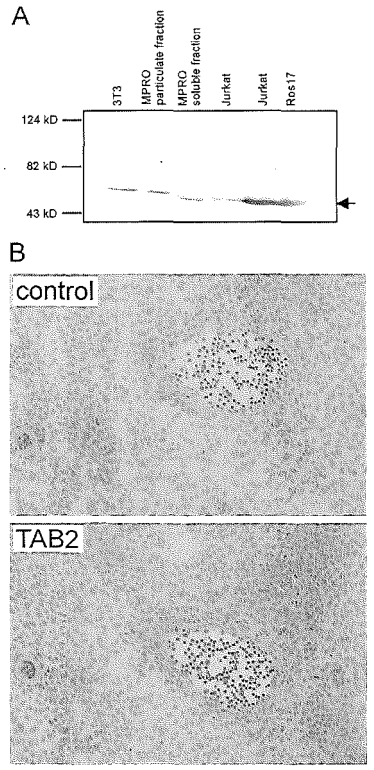

Figure 7. AGM immunostaining for mTAB2.
(A) Western blot analysis on two mouse and two human cell lines shows that the antibody raised against human TAB2 recognized a single protein band with the expected molecular mass of 77kD in both the human and mouse protein lysates. (B) Immunohistochemistry with an antibody against TAB2 was performed on 6-8μm transverse cryosections of E12 embryos at the level of the AGM. High level TAB2 expression was detected in the cells surrounding the neural tube (not shown). Lower levels of mTAB2 expression were detected in the endothelium of the E12 dorsal aorta and in some cells surrounding the dorsal aorta.

Discussion

Differential Display PCR as a means of identifying novel genes expressed differentially in embryonic tissues

To increase our understanding of the genetic program involved in the generation of the first HSCs within the mouse AGM between E10 and E11, we performed DD RT-PCR screening. When we initiated these studies, DD RT-PCR was chosen over other methods, as a non-biased means of identifying differentially expressed novel genes. In three independent DD RT-PCR screenings we found in total 42 differential bands, with 7 confirmed differentially expressed clones. In addition to the clones encoding differentially expressed genes, we also found a number of false positive clones [31]. As a known technical drawback of DD RT-PCR, excised differential bands generally contain 2-3 different PCR fragments, of which some are differentially expressed and some are not [25]. As shown in Table 2, differential band S1 yielded at least two different clones, S1-2 and S1-7, respectively corresponding to a RNA binding protein and the novel mouse *Tab2* gene. The multiple different PCR fragments present within an excised band provide an explanation for the finding that some clones have an expression pattern opposite to the original differential band (i.e. E1-2, M2-2) or do not show differential expression at all (i.e. G2-5, G4-1, K1-5, S2-6). Thus, our approach using DD RT-PCR to identify novel genes gave expected and verifiable results.

The 8 arbitrary primers we used for DD RT-PCR were sufficient to identify two novel genes differentially expressed in the E10/E11 AGM. However, for a complete overview of all the (novel or known) genes differentially expressed between two populations of cells, more primers should be used in future studies. Additionally, the recent availability of mouse E12 AGM micro-arrays holds promise for comprehensive analysis and rapid advancement in the gene expression profiles of AGM HSCs and their precursors.

Differentially expressed components of the Wnt signaling pathway are candidates for signal transduction in the AGM region.

In our screenings we found two clones corresponding to *Dvl2* and *β-catenin*, both of which are components of the Wnt signaling pathway. Generally, Wnt signals are known for their regulatory role in several developmental processes (reviewed in 8,60). As regulators of the hematopoiesis, *Wnt5a* and *Wnt10b* have been shown to increase the number of human CD34+ cells in culture and number of colonies in CFU-Mix assays [55]. *Wnt5a, Wnt10b* and several Wnt receptors (Frizzled) are expressed in E11 YS and E14 fetal liver during mouse development [1] and Wnt5a increases the proliferation of murine fetal liver hematopoietic progenitor cells [55 1]. While no obvious hematopoietic defects are found in *Wnt5a* $^{-/-}$ embryos [63], redundancy of other Wnts (i.e. *Wnt5b*) most likely plays a role.

The expression of Wnt signaling components in the AGM has not been investigated previously. In our studies we show that, besides up-regulated *β-catenin* and unchanged *Dvl2* expression, several other components of the Wnt signaling pathway including *Dvl1, Dvl3, Wnt3, Wnt5a, Tcf1, Tcf3* and *Lef* are expressed (some up-regulated at the onset of HSC activity, some unchanged) in the midgestation AGM, making it more likely that Wnt signaling may be involved in hematopoietic development. Further supporting this notion, is the finding that *Wnt3a* is expressed at E10.5 in the peri-umbilical mesenchyme, another site where HSCs are found [40].

Concerning the other Wnt signaling molecules *Tcf1, Lef* and *Tcf3*, our data on *Tcf1* and *Lef* expression correspond to previous findings of Oosterwegel et al. who reported its expression in the E10.5 to E14.5 mesonephros region and surrounding mesenchymal cells [34]. However, while *Tcf3* expression has been shown restricted to early development of the brain and become undetectable by E10.5 as determined by *in situ* hybridization [22], we were able to detect expression of *Tcf3* in the midgestation AGM and liver. This discrepancy might be due to low expression of *Tcf3* that is detectable by sensitive methods as RT-PCR but is below the detection limit of *in situ* hybridization. Altogether, the simultaneous expression of numerous Wnt signaling components in the E11 AGM and fetal liver suggest that the Wnt signaling pathway is functional. Furthermore, it is of great interest that *β-catenin*, a key component of the signaling cascade is up-regulated in the AGM within the time frame that HSCs emerge in this tissue. Thus, future studies will address the functional role of Wnt signaling in AGM cells, in particular the HSCs and the microenvironment which supports HSC induction

A conserved protein encoded by the mouse *Tm9sf2* gene is differentially expressed in the AGM region between E10 and E11.

One of the novel mouse genes, *Tm9sf2,* identified in our DD RT-PCR screening encodes a transmembrane protein. Previously, the hTM9SF2 protein was shown to localize in endosomes and was suggested to function as a channel or small molecule transporter.

Interestingly, the yeast TM9SF2 homologue is a precursor protein of which the 24kD cleavage product has been implicated in intracellular vesicle transport [3,50]. To date it is unclear whether the mouse and human TM9SF2 also require protein processing in order to fulfill their putative cellular functions.

Our interest in *Tm9sf2* arose from its up-regulated expression in the E11 AGM and because it showed some low level homology to the MDR-1 protein, which functions as a transporter in multidrug resistance and is expressed by HSCs [4,16]. However, the homology is limited to the transmembrane domain, with no clear homology with the rest of the MDR-1 protein. Additionally, the intracellular localization of the MDR-1 protein and the hTM9SF2 protein is different (cell surface versus endosomal localization respectively) and the MDR-1 protein has 12 transmembrane domains [16] while hTM9SF2 has only 9[43]. Therefore, we do not consider it likely that these proteins are closely related or within the same family of proteins. However, differential expression of *Tm9sf2* in the AGM between E10 and E11, makes this conserved gene an interesting candidate for future studies of its function and potential role in development and/or regulation or the hematopoietic system.

***Tab2*: A novel mouse gene and involvement in the IL-1 signaling cascade**

While we identified *Tab2* as a novel mouse gene in our DD RT-PCR screening of the midgestation AGM, its homologue, human *TAB2*, has been previously studied for its role in signaling within hematopoietic cells. TAB2 was originally identified as a specific IL-1 signaling component with the ability to interact with of TAK1 (TGFβ-activated kinase) [51]. TAB1, another TAK1-binding protein, was shown to be necessary for TAK1 activation upon TGFβ1, BMP and IL-1 stimulation [46,47,51]. Based on biochemical studies hTAB2 was assigned a role as adapter protein in linking TRAF6 to TAK1, but it is unclear whether it fulfills additional functions. More recently it was shown that hTAB2 is also involved in RANK signal transduction [30]. Studies in *Xenopus* have shown that TAK1 and TAB1 play a role in BMP signaling and in mesoderm patterning [46,62]. Additionally, in *Drosophila* it has been shown that TAK1 is involved in Toll signaling in the innate immune system [56]. As the Toll and IL-1 signaling pathway is conserved between *Drosophila* and vertebrates, TAB2 might also play a role in the innate immune system. Furthermore, several studies in human cell lines have shown that TAK1 and hTAB2 are involved in the activation of NFκB [12,30,37,51,61]. More recently, *Tab2* deficient mice were generated by Sanjo et al. These studies revealed that lack of *Tab2* results in an embryonic lethal phenotype due to fetal liver defects[42]. As the fetal liver is an important hematopoietic organ during development, these results strongly suggest that TAB2 either directly or indirectly is involved in the regulation of the hematopoietic system. Altogether these data indicate that in vertebrates, both TAK1 and TAB2 might play an important role in the hematopoietic system.

Of interest to our developmental studies is the fact that signaling via TAK1 and NLK negatively regulates the Wnt signaling pathway. TAK1-induced NLK activation resulted in phosphorylation of TCF/LEF factors and subsequent inhibition of β-catenin-TCF interaction with DNA. Although the biochemical studies were performed in cell lines by transient transfections, the physiological relevance of these findings was confirmed by studies in *Xenopus*, where overexpression of NLK suppressed the β-catenin-induced axis duplication [18]. Additionally, studies in *C.elegans* showed that MOM-4 (TAK1 homologue) and LIT-1 (NLK homologue) are negative regulators of TCF activity [2]. Since components

of both signaling cascades, i.e. IL-1 and Wnt, are differentially expressed in the AGM between E10 and E11, the interplay between these pathways in the midgestation AGM awaits further study.

Finally, the upregulation of *Tab2* in the AGM coincides with the emergence of HSCs within this region and parallels the presence of HSCs in the fetal liver. As shown by immunohistochemical staining, TAB2 expression is localized to the endothelium of the dorsal aorta and in some underlying mesenchymal cells in the E11 AGM. Previous studies in our laboratory, have shown that HSCs are located within the endothelium of the E11 dorsal aorta and in the closely associated mesenchyme [10,11]. Our preliminary investigations of mTAK1 expression in the AGM, show that mTAK1 expression is also confined to endothelium and the underlying mesenchymal cells. Based on the spatial and temporal expression pattern of *Tab2*, we suggest that *Tab2* is involved either directly or indirectly in the induction and/ or regulation of HSCs within the AGM. Currently it is unclear whether mTAB2 is involved in IL-1, RANK or another not-yet-identified signaling cascade. Future studies should yield more insight into the precise role played by *Tab2* and its potentially exciting role in AGM HSC development.

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Appendix: Results DD RT-PCR screenings

screen	clone	Expr on gel (*)	Expr. Profile dot blot screen / RT-PCR (*)	Homology to gene product	Homology to accession number	Remarks
1	D1-1	↑	ND	mouse EST	AK007682	homologous to human myeloid/ lymphoid or mixed-lineage leukemia 5
1	D2-3	↓	ND -			
1	E1-2	↓	↑	human TM9SF2	NM004800	new mouse gene
1	G1-3	↓	↓	mouse Enolase/ myc binding protein (MBP)	BC024644	
1	G2-4	↓	ND	mouse tubulin	NM_011653	
1	G2-5	↓	similar	mouse BUB1B	AF107296	
1	G3-4	↑	ND	mouse EST	BC004697	
1	G4-1	↓	similar	mouse Dvl2	NM007888	
1	G4-7	↓	ND	mouse nucleosome assembly protein 1-like 1	NM_015781	
1	H1-3	↑	↓	mouse Enolase/ myc binding protein (MBP)	BC024644	
1	H2-7	↑	ND	mouse fibronectin	XM_129845	
1	I1-5	↑	ND	mouse EST	XM_129565	
1	I1-7	↑	ND	vector sequence	AF173954	
1	I2-8	↑	ND	mouse EST	BC019518	
2	K1-5	↑	similar	mouse Cu/Zn superoxide dismutase	X06683	
2	K1-6	↑	↑	mouse globin gamma/ epsilon	M26897	
2	K2-1	↑	ND	mouse Sjogren syndrome antigen b	NM-009278	RNA binding protein
2	K2-8	↑	ND	mitochondrial DNA	J01420	DNA contamination
2	K3-7	↑	ND	Rb binding protein Rbbp4/ mRbAp48	XM_109424	
2	L1-1 L1-5	↓	ND	Xist	XM_136007	
2	L2-3 L2-8	↓	ND	Xist	XM_136007	

screen	clone	Expr on gel (*)	Expr. Profile dot blot screen / RT-PCR (*)	Homology to gene product	Homology to accession number	Remarks
2	M1-1 M1-4	↑	ND		No homology	
2	M2-2	↓	similar	mouse TRAP150	NM005119	
2	M2-7	↓	ND	rat/human lipoprotein binding protein	U90725	new mouse gene
2	N1-1 N1-8	↑	ND	mouse SMT3 homolog 2	XM_122275	
2	N2-2	↑	ND	genomic DNA		DNA contamination
2	N2-6	↑	ND	ribosomal protein		
2	N3-1 N3-7	↑	ND	ribosomal protein		
2	N4-2	↑	ND	mouse EST	XM_129041	similar to hypothetical protein BC014003
2	N4-8	↑	ND	ribosomal protein		
2	N5-2	↑	ND	ribosomal protein		
2	N5-8	↑	ND	genomic sequence		DNA contamination
2	P1-1 P1-5	↑	ND	zinc RING finger protein SAG	AF092877.1	
2	Q1-2 Q1-5	↑	ND	mouse similar to Elongation factor 1-gamma	XM_124966	
2	Q2-1	↑	ND			no homology
2	Q2-2	↑	ND	peptidylprolyl isomerase C	NM_008908	
3	R1-3	↓	ND	human Rb binding protein	AK008164	new mouse gene?
3	R1-4	↓	ND	mouse homolog to 60S ribosomal protein L4 (L1)	AK012432	
3	R2-3	↑	ND	mouse mitochondrial DNA	J01420	DNA contamination
3	R2-5	↑	ND		no homology	
3	S1-1	↑	ND		no homology	
3	S1-2	↑	similar	homolog of human ftp-3	NM019868	RNA binding protein highly expressed in E11/E12 liver
3	S1-7	↑	↑	human TAK-1 binding protein	AF241230	IL-1R signaling, new mouse gene

screen	clone	Expr on gel *	Expr. Profile dot blot screen / RT-PCR *	Homology to gene product	Homology to accession number	Remarks
3	S2-4	↑	↑	β-catenin	NM_007614	Wnt signaling
3	S2-6	↑	similar	EST	BG071065	
3	T1-5	↑	ND	human zinc finger protein 339	NM_021220	low homology
3	T1-8	↑	ND	mouse fibrillin 2	NM_010181	
3	T2-1 T2-2	↑	ND	human zinc finger protein 339	NM_021220	low homology
3	T3-1 T3-4	E11 liver	ND	human DNA		low homology
3	V1-1 V1-8	↑	ND	similar to chromodomain helicase DNA binding domain	XM_132858	
3	V2-5	↑	ND	genomic DNA		DNA contamination
3	V2-8	↑	ND	hypothetical protein XP_155909	XM_155909	
3	V3-4	↑	ND		no homology	
3	V3-8	↑	ND	mouse Btk locus	U58105	
3	V4-2	↑	ND		no homology	
3	V4-5	↑	ND	mouse DNA clone RP23-3D10	AC098723	DNA contamination
3	V5-1	↑	ND		no homology	
3	V5-6	↑	ND	human DNA clone RP11 182B22	AI359259	DNA contamination

(*) transcripts that are expressed at higher levels in E11 aorta/ AGM tissue in comparison to E10 AGM are referred to as upregulated (indicated by ↑) and transcripts that are expressed at lower levels in E11 aorta or AGM tissue in comparison to E10 AGM are referred to as downregulated (indicated by ↓). ND is non-determined.
In Differential Display RT-PCR screening 1 complete E10 AGM was compared to complete E11 AGM material. In screening 2 E10 aorta was compared to E11 aorta material and in screening 3 complete E10 AGM was compared to E11 aorta and E11 UGR material.

Chapter 3

Expression of TAB2, a TAK1 binding protein in adult mouse tissues

Claudia Orelio, Karin van der Horn, Esther Haak, Elaine Dzierzak

Abstract

TAK1, a MAPK member, and TAK1 binding (TAB) proteins are highly conserved between several species. TAK1 has been implicated as a component of BMP, Wnt, IL-1, RANK and other signaling pathways and TAB1 was shown to be crucial for the activation of TAK1. In addition to TAB1, two other TAB proteins, TAB2 and TAB3 are similarly thought to act as adapter proteins. However, little is known about the role and regulation of TAB2 and TAB3. As the expression pattern could reveal more insight into the function of these molecules we analyzed the expression of *Tab2* and *Tab3* at the RNA level and also investigated TAB2 protein expression in the mouse. We found *Tab2* and *Tab3* transcripts in several adult tissues and cell lines and show that the *Tab2* and *Tab3* expression pattern partially overlaps. Furthermore, we report TAB2 protein expression in several adult tissues, including the thymus, spleen, brain, lung and bone marrow. Interestingly, the TAB2 protein expression pattern shows similarities to reported pattern of NFκB activity suggesting a functional link.

Introduction

Mitogen activated protein 3 kinase 7 (MAP3K7) is better known as Transforming growth factor β (TGF-β) activated kinase 1 or TAK1. As suggested by its name, TAK1 was originally identified in a screen for proteins involved in TGF-β signaling[39]. In the adult, TAK1 is expressed ubiquitously, with highest expression levels in thymus and brain[39]. During development, TAK1 expression at E10.5 was observed in the spinal cord and the brain. From E12.5 onwards high levels of TAK1 expression were observed in the gut epithelium and the mesonephric and metanephric kidneys. TAK1 expression was also detected in E14.5 testis, lung and pancreas[14].

Soon after the identification of TAK1, the identification of two binding proteins, TAB1 and TAB2 was reported[31]. TAB1 is ubiquitously expressed and constitutively associated with TAK1. As overexpression of TAB1 resulted in enhanced TAK1 kinase activity, it was proposed that TAB1 is a positive regulator of TAK1. Later it was shown that a conserved C-terminal α-helix motif in TAB1 is involved in the binding and activation of TAK1[23]. Apart from limited Northern and RT-PCR RNA analysis, the temporal expression pattern of *Tab1* during adult and embryonic stages in the mouse has not been determined to date. However, *Tab1* deficiency leads to perinatal lethality due to lung and cardiovascular abnormalities, indicating an important role for TAB1 in the development of these tissues[18].

Both TAK1 and TAB1 are highly conserved, and homologous proteins have been identified in human, mouse, *Xenopus*, *Drosophila* and *C.elegans*. The TAK1 and TAB1 homologues in *C.elegans* (MOM-4 and TAP-1) were shown to antagonize Wnt signaling during development[21,36]. In mammalian cells and during *Xenopus* development a similar antagonizing effect of TAK1 was shown to downregulate Wnt induced β-catenin/ TCF transcriptional activation[10-12]. Moreover, TAK1 and TAB1 were implicated in BMP signaling during *Xenopus* development[30,38]. Further investigations revealed that BMP-4 could activate TAK1 and thereby exert a negative effect on neural specification in *Xenopus* development[6]. Additionally, TAK1 has been implicated in several other signaling pathways including the interleukin (IL-1) and lipopolysaccharide (LPS) signaling pathways[9,35].

Apart from the relatively well characterized TAB1 protein, two other TAB proteins have been identified, TAB2 and TAB3. In contrast to TAB1 which has a clear role

in TAK1 regulation, the role of TAB3 and to a lesser extent TAB2 in TAK1 regulation remains unclear. TAB2 was originally reported as a adapter protein required for interaction of TAK1 and TRAF6 in IL-1 receptor signaling[34]. Biochemical protein analysis revealed that upon IL-1 stimulation both TAK1 and TAB2 are translocated from the plasma membrane to the cytosolic fraction. This is consistent with the idea that TAK1 and TAB2 would interact with the IL-1 receptor prior to transducing signals to other proteins[16,34,35]. However, recent studies by Beak et al. showed that TAB2 is localized in the nucleus of neuronal cells and translocated to the cytoplasm upon IL-1 stimulation[1]. They proposed that TAB2 in the nucleus fulfilled a role as modulator of NFκB-mediated gene activation[1]. Further evidence that TAB2 might not play a crucial role as an adapter protein in IL-1R signaling, was provided by *Tab2* gene disruption experiments[27]. *Tab2* deficient mice die around E12.5 due to fetal liver degeneration. Upon IL-1 stimulation, *Tab2*-deficient cells responded similarly to wildtype cells in the expression of IL-1 target genes and the activation of JNK and NFκB[27]. Thus, the exact role of TAB2 in IL-1 signaling remains controversial. The last identified TAB protein, TAB3 was shown to be involved in BMP signaling in Xenopus. Based on its high homology to TAB2, it was proposed to function as an adapter protein linking TAK1 to the BMP receptor.

Currently, little information is available about the expression of TAB2 and TAB3. As these proteins are highly homologous and therefore may fulfill overlapping functions we examined the expression pattern of *Tab2* in the adult mouse and compared it to that of *Tab3*. Here we show that both *Tab2* and *Tab3* are ubiquitously transcribed in most adult mouse tissues examined, although *Tab3* is expressed at lower levels than *Tab2*. Moreover, by protein analysis we show TAB2 expression in certain cell subsets in the brain, spleen, thymus and in the bone marrow.

Material and Methods

Mice 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. (CBAxC57/BL10)F1 Mice were sacrificed by cervical dislocation and tissues collected in PBS/10%FCS.

3T3 fibroblasts were maintained in DMEM/ 10% FCS and transiently transfected with Lipofectamine2000 (Life Technologies) according to manufacturers instructions and harvested 24 hours after transfection.

RNA expression analysis by RT-PCR and Northern blotting

Total RNA was isolated with TRIZOL (Gibco/ Life Technologies) according to manufacturer's instructions and DNAse treated (RQ1 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 min 92°C, 30-35x (40 sec 92°C, 40 sec 58°C, 1 min 72°C), 7 min 72°C. PCR primers are listed in Table 1.

For Northern analysis 10-20μg total RNA was separated on a formaldehyde gel and blotted to Hybond-N membrane (Amersham Pharmacia Biotech). Probes were labeled with ^{32}P by nick translation method (Amersham Pharmacia Biotech). *Tab2* probe contained the complete open-reading frame sequence and *Tab3* probe consisted of fragment 731-1540 based on sequence XM-111986.

Table 1. RT-PCR primers

Primer	Sequence (5'-3')	product size	accession number
β-actin forw	CCTGAACCCTAAGGCCAACCG	397	X03672
β-actin rev	GCTCATAGCTCTTCTCCAGGG		
IL-1RacP forw	GAAATAGCCTCAGCTCACACAG	458	NM_008364
IL-1RacP rev	CTCTGTTCCACCTCAGACTC		
IL-1RI forw	ACCTCACTTCTCCTGGATCC	300	NM_008362
IL-1RI rev	GGGGTACAAAGAACAAGGCG		
IL-1RII forw	CCAGCATCATTGGGGTCAAG	435	NM_010555
IL-1RII rev	CCTGGTTGTCAGTCCGTAGC		
TAB1-910 forw	CAGGCCAACCAGGATTGC	454	NM_025609
TAB1-1360 rev	GAGCTGGAGCTGCTGCTCTG		
TAB2-1680 forw	TGCTGGTGCACCAGAAGGCCAGGATG	686	BC004813
TAB2-2350 rev	GTCTCTCAGGCCTTTCAGGTG		
TAB3 forw	CTCAGAATGCTCCATGG	809	XM_111986
TAB3 rev	ATCCAGAACTAGAGCTC		
TAK1 (3') forw	GTGGTACATTACAGAGGGAC	412	BC006665
TAK1 (3') rev	CCGATAGCTCAGCTCAAGCC		

Western blotting

Protein lysates were made with RIPA lysis buffer. Proteins were separated on 10% SDS-PAGE and blotted to PVDF membrane (Immobilon, Millipore). Blotted membranes were blocked with 4% non fat dry milk (Biorad) /TBS-T and incubated overnight with primary antibody in 2%milk/TBS-T, then incubated with HRP labeled secondary antibody and visualized by ECL detection. Antibodies used were TAB2 (against N-terminus, k-20; Tebu Bio), TAB2 (against C-terminus, PA1-853 Affinity BioReagents) and α-HA (Santa Cruz).

MACS sorting for hematopoietic lineage negative and positive BM cells

BM from adult mice was collected in PBS/ 10% FCS and stained with antibodies against CD11b, CD5, Gr1, Ter119, B220 (BD Pharmingen and Immunotech) for 30 min on ice. Subsequently cells were incubated with goat-anti rat microbeads (Miltenyi Biotec). Lineage negative and positive cells were separated with the autoMACS system (Miltenyi Biotec). Purity of cell fractions was checked by FACS analysis and the lineage negative fraction had a purity of 80-85%.

Immunofluorescence and histochemistry

Adult tissues were snap-frozen and 7-10μM cryosections were made. Sections were fixed in 2% paraformaldehyde/ PBS and incubated with primary antibody overnight at 4°C. Staining was visualized with Santa Cruz or Vector Laboratories ABC staining kit in combination with TSA biotin system (PerkinElmer) according to provided instructions. Sections were counterstained with hematoxylin, dehydrated and embedded in Entallan.

For immunofluorescence studies cytospins of BM cells were made. Cells were fixed and stained as described above. Staining was visualized with a secondary FITC labeled antibody.

Results

TAK1 binding proteins are conserved between several species

To date three different TAK1 binding proteins have been described, TAB1, TAB2 and TAB3. These TAK1-binding proteins are highly conserved between several species including *C.elegans*, *Xenopus*, mouse and human with approximately 90% homology between mouse and human. As shown in Figure 1A, TAB1 is the smallest (approximately 500 amino acids) of the TAK1 binding proteins and contains a conserved phosphatase domain, although to date no phosphatase activity has been observed for TAB1. TAB2 and TAB3 are also highly conserved between several species with approximately 90% homology between mouse and human. The protein structure of TAB2 and TAB3 is similar and these proteins are larger than TAB1. Both TAB2 and TAB3 contain an N-terminal CUE domain, a C-terminal zinc finger domain and a coiled coil domain. The CUE domain has been suggested to play a role in ubiquitination and possible degradation or regulation of these proteins[17]. The coiled coil domain most likely fulfills a role in protein-protein interactions and the zinc finger domain might also be involved in protein-protein interactions, although DNA-protein interaction cannot be excluded. In addition, TAK1 is highly conserved at the protein level between human and mice (95%) and to a somewhat lower extent with *Xenopus* (data not shown). TAK1 contains a conserved kinase domain and a coiled coil region, which most likely plays a role in protein-protein interactions (Figure 1A).

As described by Baek et al., TAB2 is localized in the nucleus of neuronal cells and is translocated to the cytoplasm upon IL-1 stimulation[1]. A nuclear export signal (NES) within the coiled coil domain of TAB2 was detected. Site-directed mutagenesis of this domain resulted in constitutive localization of TAB2 in the nucleus[1]. Protein sequence comparison reveals that a similar NES sequence is present in mouse and human TAB3 (Figure 1B) suggesting that TAB3 might also be localized in the nucleus and regulated in a similar fashion to TAB2. Furthermore, as shown in the protein sequence alignment mouse, human and *Xenopus* TAB2 and TAB3 are highly homologous with approximately 46% overall protein similarity (Figure 1C). Highest similarity (~80%) is found at the C and N-terminus of these proteins. As both TAB2 and TAB3 have similar domains and both contain a NES sequence, it is possible that the localization of these proteins is similar and that they have redundant or overlapping functions.

TAB1, 2 and 3 and TAK1 expression in mouse cells

TAB2 was originally identified as a binding partner of TAK1 and was proposed to fulfill an important role in IL-1 signaling[34]. Previously, we reported *Tab2* RNA expression in the midgestation AGM and liver and in several adult mouse tissues, including the thymus, spleen and BM [24]. Additionally, TAB2 expression has been observed in neuronal cells[1]. To investigate whether the different TAK1 binding proteins are co-expressed in adult mouse tissues, we performed RT-PCR analysis. As shown in Figure 2A, *Tak1* is ubiquitously expressed in all tissues examined. *Tab2* is expressed at high levels in liver, kidney, stomach and spleen. *Tab1* and *Tab3* are expressed in these same tissues, but at much lower levels than *Tab2* (35 RT-PCR cycles were necessary to detect expression of TAB1 and 3 as compared to 30 cycles for TAB2). Thus, *Tak1* and *Tab1, 2* and *3* transcripts are co-expressed in several adult tissues.

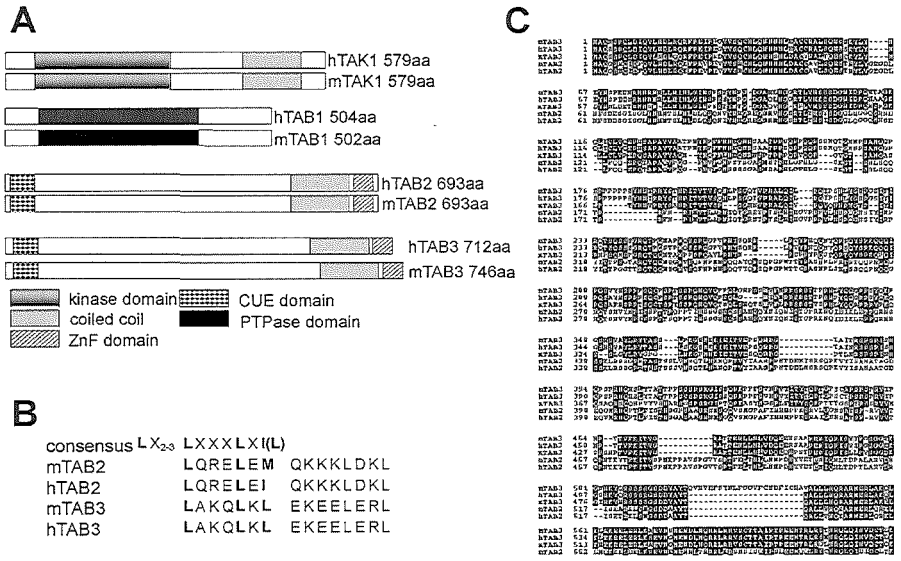

Figure 1. Protein structure and conservation of TAK1 and TAB proteins
(A) TAK1 and TAB protein domains are similar between mouse and human. Conserved protein domains in TAK1 are the N-terminal kinase domain and the C-terminal coiled coil domain. In TAB1 a conserved phosphatase (PTPase) domain is present. TAB2 and TAB3 contain similar domains; an N-terminal CUE domain, a C-terminal coiled coil domain and Zinc finger (ZnF) domain. aa= amino acids. (B) Alignment of the NES of mouse and human TAB2 and TAB3 is shown. (C) Alignment of mouse, human and Xenopus TAB3 with mouse and human TAB2 protein reveals high homology at the protein level of related proteins. The highest similarity of TAB2 and TAB3 is found at the C-terminus and the N-terminus of the proteins.

Tab2 and *Tak1* transcripts have been found in hematopoietic cell populations in the BM [13,25]. To examine the hematopoietic expression pattern of the *Tak1* and more specifically of all three *Tab* genes in the bone marrow (BM), mature hematopoietic lineage negative and positive cells were sorted by MACS, followed by RT-PCR analysis. The lineage negative fraction of the BM is enriched for hematopoietic stem and progenitor cells, while the lineage positive fraction is enriched for the mature types of hematopoietic cells. As we had detected *Tab2* and *Tak1* expression in the 416B hematopoietic precursor cell line in other experiments, cDNA from this cell line was used as a positive control for the RT-PCR reaction. As shown in Figure 2B, expression of *Tak1* and all three *Tabs* was observed in both BM fractions, with the tendency towards higher expression in the lineage negative fraction. Since TAB2 has been implicated in IL-1 signaling, we also tested expression of the IL-1 receptor in the two BM fractions. Expression of the two signaling IL-1 receptor subunits (*Il-1rI* and *Il-1racp*) and the non-signaling IL-1 receptor (*Il-1rII*) was observed in both BM fractions.

To quantify more accurately the expression levels of *Tab2* and *Tab3* we performed Northern Blot analysis. To minimize cross-reactivity, we used as a *Tab3* probe a fragment with low nucleotide homology to *Tab2*. As shown in Figure 2C, both *Tab2* and *Tab3* overlap in expression in brain, kidney, thymus, spleen and BM. When, we investigated

Tab2 and *Tab3* RNA expression in several cell lines, the highest expression for *Tab2* was observed in myeloid (DA3 and 32D) and 3T3 fibroblast cell lines, while low *Tab3* expression was detected in most other cell lines. While it is clear from these data that the *Tab2* and *Tab3* expression patterns are similar in adult mouse tissues, the expression pattern and levels in several cell lines is very different. Moreover, *Tab3* is expressed at lower levels than *Tab2*.

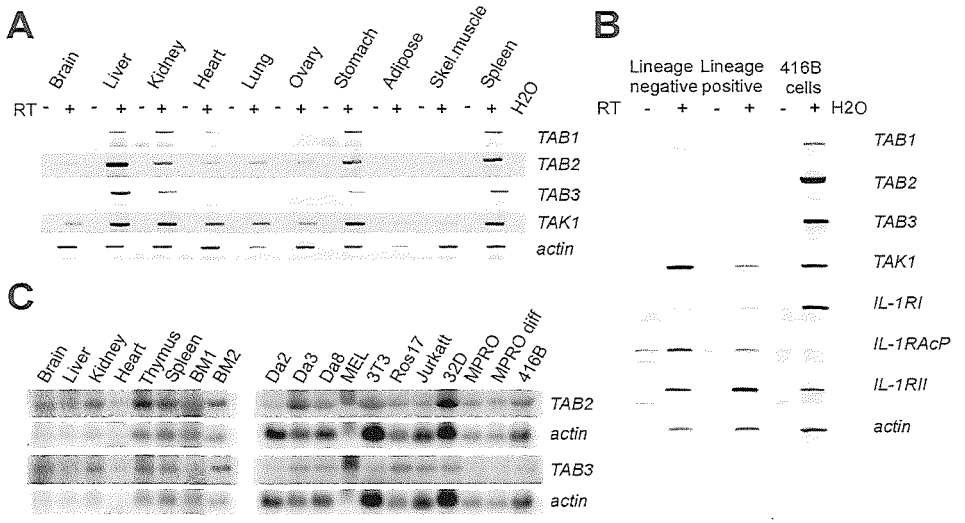

Figure 2. Tak1 and Tab genes are expressed ubiquitously in mouse tissues and cells.
(A) RT-PCR expression analysis for Tak1 and Tab genes in several adult mouse tissues. The expression pattern for Tak1 and the Tab genes is more or less similar, with high expression in the liver, kidney, heart, stomach and spleen. (B) RT-PCR expression analysis of Tak1, Tab genes and components of the IL-1 receptor in MACS-sorted lineage marker negative and positive cells from adult bone marrow (n=2). As positive control for the RT-PCR the hematopoietic 416B cell line was used. All genes tested are expressed in both the lineage negative and positive bone marrow fraction. (C) Northern blot analysis for Tab2 and Tab3 on several adult mouse tissues (left panel) and mouse and human cell lines (right panel). Tab3 and Tab2 tissue expression patterns are similar. Tab3 expression is generally lower than Tab2 expression. In the panel of the cell lines Tab2 expression is highest in myeloid cells (DA3 and 32D) and 3T3 fibroblasts. Expression of Tab3 is low in all cell lines tested. The Tab3 probe hybridizes to a transcript larger than that of Tab2, consistent with the identification of longer Tab3 cDNA sequences (approximately 8 kb; sequence AL832071) than those for Tab2 cDNA (approximately 3.5kb; sequence BC004813). Actin is used as a RNA normalization control. BM=bone marrow, RT=reverse transcriptase.

TAB2 protein expression in the mouse

Previously, we reported mTAB2 expression near and in the endothelium of the dorsal aorta at E11 and E12 of mouse development [24]. However, the expression pattern of TAB2 in the adult has to date not been investigated. To investigate the protein expression of mTAB2 in adult mouse tissues we performed Western blot analysis.

In order to confirm the molecular weight of the TAB2 protein, which was claimed to be 76 kD, we overexpressed a HA-tagged full length TAB2 (Figure 3A) construct in 3T3 fibroblast cells. As a positive control for the α-HA antibody we used a HA-tagged Oct6 protein lysate. As shown in Figure 3B, the protein lysate containing the HA-TAB2 full length protein yielded a band on Western with a molecular weight of approximately 75-80 kD, consistent with the expected size of ~79 kD (76 kD plus 3 kD) of the TAB2 protein with the HA-tag. Additionally, overexpression of the truncated HA-TAB2 construct yielded a smaller protein product on Western blot. To confirm that the protein bands as detected

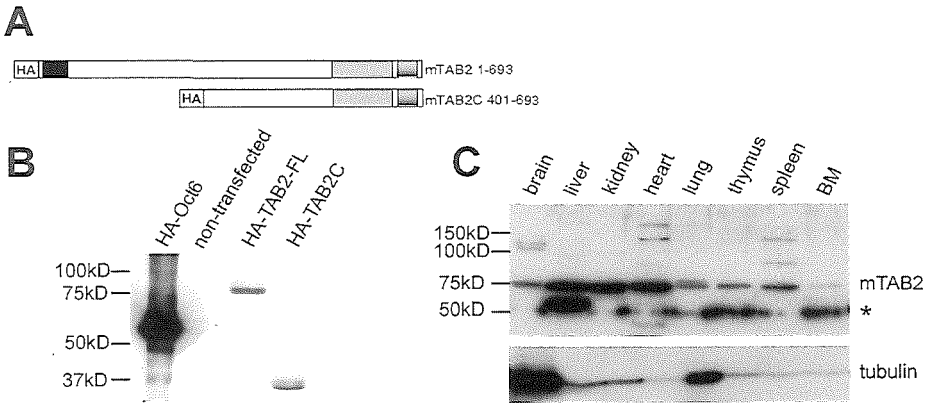

Figure 3. TAB2 protein analysis in the adult mouse by Western blotting
(A) The HA-TAB2 full length and HA-TAB2C constructs were used for overexpression in 3T3 fibroblasts. These constructs were used as controls to determine the molecular weight of the TAB2 protein (B) Western blot containing protein extracts of 3T3 fibroblast cells transfected with HA-TAB2 constructs (as indicated in Fig.3A) was incubated with an α-HA antibody and shows that the HA-TAB2 full length protein has a molecular weight of 75-80kD and that the truncated HA-TAB2C construct has a molecular weight of ~35kD. HA-Oct6 protein extract was used as a positive control for the α-HA antibody. (C) Western blot containing protein extracts from several adult mouse tissues incubated with TAB2 antibody directed against the C-terminus of TAB2 (Affinity BioReagents). A protein band at ~75kD corresponds to the molecular weight band of the HA-TAB2 full length protein and therefore likely represents TAB2. The highest TAB2 expression is found in liver, kidney and heart. Lower TAB2 expression is found in the hematopoietic organs; bone marrow (BM), thymus and spleen. The asterisk (*) indicates an aspecific band. As a control for protein loading the blot was reprobed with α-tubulin antibody.

with the α-HA antibody represented TAB2 proteins, we incubated the blot with an α-TAB2 antibody. Despite the fact that an endogenous TAB2 band was readily detected, we were unable to detect a slightly larger HA-TAB2 protein. Most likely, this is due to the very low expression level of the HA-tagged TAB2 constructs. Alternatively, the N-terminal HA-tag might have interfered with the recognition of HA-TAB2 by the α-TAB2 antibody that is directed against the N-terminal part of TAB2. Nevertheless, when a Western blot containing protein lysates of adult mouse tissues was probed with a α-TAB2 antibody we detected a protein band with a molecular weight of approximately 75 kD (Figure 3C). Based on the protein size as detected in the Western with HA-tagged TAB2, it is highly likely that this band corresponds to TAB2. High expression of the 75 kD TAB2 band was

observed in brain, liver, kidney, heart and lung and low levels were observed in the hematopoietic tissues, thymus, spleen and BM (Figure 3C). Besides the 75 kD band another band of approximately 55-60 kD was detected by Western blotting with the TAB2 antibody raised against the N-terminal part of TAB2 (data not shown). It is unlikely that the 55-60 kD band represents the highly homologous TAB3, as the predicted TAB3 protein has a higher molecular weight than TAB2. As this 55-60 kD band was not detected by the antibody directed against the C-terminal part of TAB2, this 55-60 kD band could represent a C-terminal truncated TAB2 isoform.

Localization of TAB2 in adult mouse tissues

To more specifically identify and localize the cells expressing TAB2 in adult mouse tissues, we performed immunohistochemistry. Cryosections of several adult mouse tissues, including brain, thymus, spleen, lung, kidney, heart and liver were incubated with TAB2 specific antibodies and visualized with the peroxidase substrate DAB chromogen. For these analyses we used two different TAB2 antibodies (directed against the N- and C-terminus of TAB2) and both antibodies gave similar TAB2 expression patterns in the tissues tested. Sections were counterstained with hematoxylin to visualize nuclei of the cells in the tissue, as an indication of the overall morphology of the tissues.

In the coronal adult brain sections TAB2 expression was observed in cells scattered throughout the grey matter of the cerebellum. High expression was observed in cells in the outer layers of the cortex, excluding the outermost layer (Figure 4A-C). The cells underlying the outermost layer of the cortex are mainly neuronal cells and almost all these cells expressed TAB2. In general the highest percentage of TAB2 expressing cells was localized immediately beneath the outermost layer of the cortex. Interestingly, TAB2 staining overlapped with the nuclear hematoxylin staining, suggesting that TAB2 in neuronal cells in the adult brain is localized mainly in the nucleus.

The spleen is a hematopoietic tissue that is comprised of white pulp and red pulp areas. The white pulp area of the spleen harbors mainly lymphocytes while the red pulp area harbors mostly erythrocytes and connective tissue. The white pulp areas are separated from the red pulp by the marginal zone, where also marginal zone macrophages are located [19,20]. Blood cells enter the spleen via the trabecular arteries and leave the circulation via the central arterioles. Blood cells re-enter the circulation via the sinusoids and the veins. In the spleen, we observed TAB2 expression in the endothelium of the blood vessels, most likely the central arterioles and the trabecular arteries, but not in the sinusoids (Fig. 4D-F). Moreover, TAB2 expression was observed in a few scattered cells (less than 1%) throughout the spleen. Most of these TAB2 expressing cells were located near blood vessels or at the border of the white and red pulp areas, the marginal zone. As the marginal zone is known to contain macrophages, these TAB2 expressing cells could represent a (sub) population of marginal zone macrophages.

Similar to the spleen, the thymus is an important lymphoid tissue. It can be separated into the outer cortex and the inner medulla area. In the thymus, the less differentiated lymphocytes are localized to the cortex, while more mature differentiated lymphocytes are localized to the medulla area. Similar to the spleen, TAB2 expression in the thymus was observed in the endothelium of blood vessels. Additionally, a low percentage of cells scattered throughout both the cortex and the medulla regions expressed

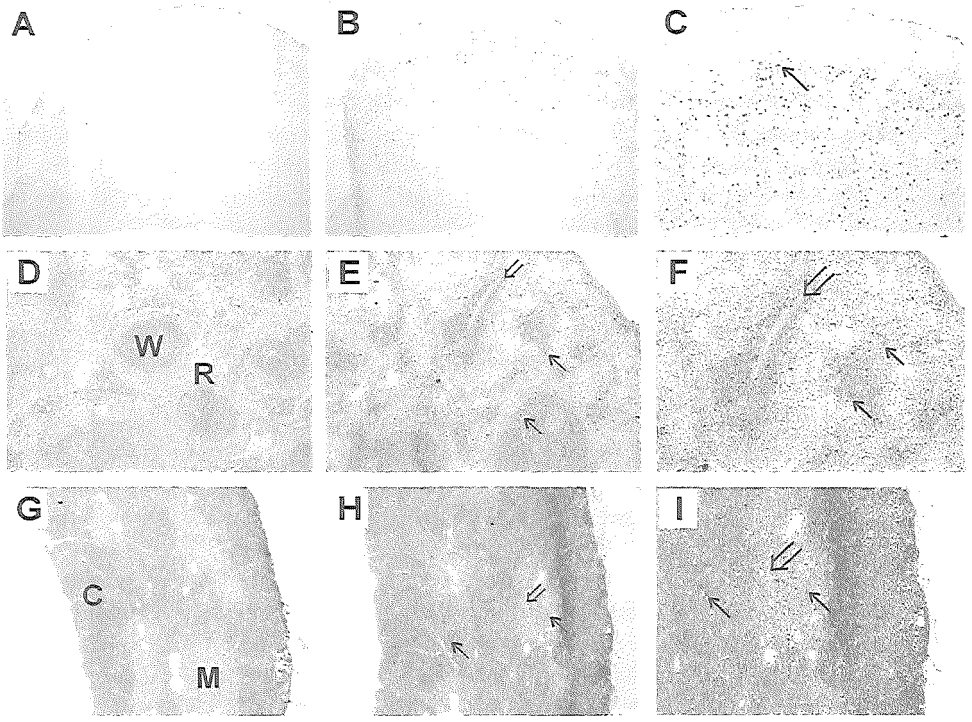

Figure 4. TAB2 expression in adult mouse tissues.
Cryosections of the brain, spleen and thymus stained with TAB2 (k-20; Santa Cruz) antibody. Tissues are counterstained with hematoxylin. (A-C) brain (D-F) spleen (G-I) thymus. (A,D,G) negative control; staining without TAB2 antibody (B,C,E,F,H,I) TAB2 staining (A-C) In the brain TAB2 expression can be observed in neuronal cells in the outer layers of the cerebellum, except for the outermost layer where no TAB2 expression is observed. TAB2 is localized to the nuclei of the cells. (D-F) In the spleen TAB2 staining is detected in the endothelium of blood vessels and in few scattered cells at the borders of the red and white pulp area and near blood vessels. W=white pulp area and R=Red pulp area. (G-I) In the thymus TAB2 expression is located to the endothelium of blood vessels and few scattered cells in both the cortex as the medulla area. C=cortex and M=medulla. In (C,E,F,H,I) the closed arrows indicate TAB2 expressing cells and the open arrows indicate endothelial cells in the blood vessels expressing TAB2

TAB2 (Figure 4G-J). As the amount of TAB2 expressing cells in the thymus is very low, it is not likely that these cells represent lymphocytes, as lymphocytes are abundant in the thymus. Since the TAB2 expressing cells in the spleen, based on their size and localization, could represent a (sub) population of macrophages, the TAB2 expressing cells in the thymus might also represent (a subpopulation of) macrophages. This would be consistent with the observation that in both tissues of low numbers of TAB2 expressing cells are present and that the TAB2 expressing cells appear to be rather large cells. Interestingly, in contrast to the brain where the intracellular localization of TAB2 appeared to be nuclear, in the spleen and thymus TAB2 expressing cells, TAB2 staining surrounded the hematoxylin staining. This indicates that in spleen and thymus cells the TAB2 protein is cytosolic.

Besides TAB2 expression in the brain, spleen and thymus, we also observed TAB2 expression in the endothelium of blood vessels and low levels of TAB2 expression in the endothelium of some bronchioli in the lung. Additionally, in the E12 developing lung TAB2 expression was detected in a layer of cells underlying the lung epithelium and in the pulmonary arteries (data not shown). In the heart no clear TAB2 expression was observed throughout the myocardial tissue, but clear TAB2 expression was observed in the endothelium of blood vessels and in some cells located near blood vessels (data not shown). For the liver and kidney tissues examined, we were not able to detect specific TAB2 expression due to high background staining. We cannot exclude the possibility that TAB2 is expressed at low levels in these tissues.

In conclusion, TAB2 expression in the adult mouse is observed in endothelial cells of blood vessels in several tissues and in neuronal cells in the brain. Furthermore, few scattered cells in the hematopoietic/lymphoid tissues, the spleen and thymus, expressed TAB2. Based on their localization in the spleen and their size in these tissues, these cells could represent a (sub) population of macrophages.

TAB2 is localized in the nucleus and cytoplasm of bone marrow cells

To more specifically determine the intracellular localization of TAB2 we performed immunofluorescence analysis on cytospins of adult bone marrow cells with an α-TAB2 antibody followed by a fluorescent secondary antibody. Since we found *Tab2* transcripts in both the lineage negative and positive fraction of the bone marrow, we examined both populations for TAB2 protein expression and intracellular localization. In both BM cell fractions we were able to observe TAB2 protein expression (Figure 5 E/F). In most cells examined, the staining for TAB2 was similar to the nuclear DAPI staining (Figure 5 A/B/C), indicating that TAB2 is localized in the nuclei of these cells. However, in some cells also cytoplasmic TAB2 localization was observed.

The size and shape of the nucleus together with the ratio between the nucleus and the cytoplasm allows discrimination between several different cell types in the hematopoietic system. Based on the nuclear shape and size as observed by the DAPI staining together with the cytoplasmic TAB2 staining (in some cells), we were able to distinguish some hematopoietic cell types in these cytospins of these bone marrow cells. Mature myeloid cells (granulocytes) have a doughnut shaped nucleus and can be easily distinguished. Immature myeloid cells have large and bean-shaped nucleus, while hematopoietic progenitor (blast) cells tend to have a small round nucleus and relatively small cytoplasm. As shown in Figure 5, within the lineage positive BM fraction both immature and mature myeloid cells are observed. The immature myeloid cells show high levels of TAB2 in the nucleus and the mature myeloid cells have low levels of TAB2 in the nucleus and higher levels of TAB2 in the cytoplasm. In the lineage negative BM fraction, some mature myeloid cells have TAB2 localized both in the nucleus and cytoplasm. Additionally, the hematopoietic progenitor (blast) cells in the lineage negative fraction express high levels of TAB2 in the nucleus. Hence, in contrast to mature myeloid cells, hematopoietic progenitor cells and immature myeloid cells express higher levels of TAB2 with a predominant localization in the nucleus.

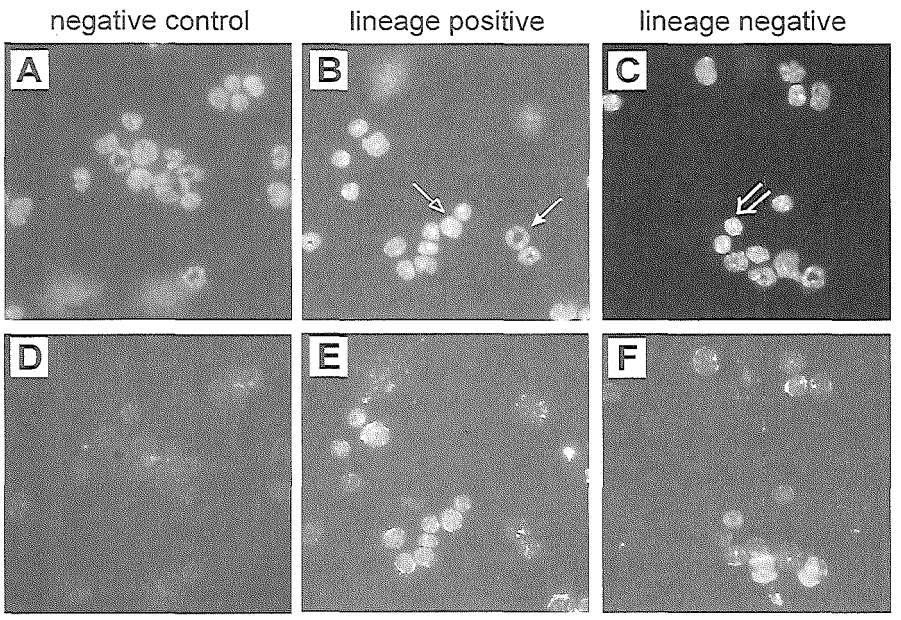

Figure 5. Intracellular TAB2 localization in adult bone marrow cells.
Adult bone marrow cells were sorted into a lineage marker positive and negative fraction by MACS and cytospins were made. Cells were fixed and processed for immunofluorescence staining with a TAB2 (Affinity BioReagents) antibody. (A)(B)(C) show nuclear DAPI staining (D)(E)(F) show TAB2 staining. (A)(D) negative control for TAB2 staining; (B)(E) lineage positive fraction (C)(F) lineage negative fraction. In both the lineage negative and positive fractions TAB2 expressing cells are present and TAB2 staining is present in both the nucleus as well as the cytoplasm. Arrow with closed tip indicates a mature myeloid cell, arrow with open tip indicates immature myeloid cells and the open arrow indicates hematopoietic progenitor (blast) cells.

Discussion

TAK1 and TAK1 binding proteins are conserved between several species

TAK1 and TAK1 binding proteins are highly conserved between several species. The similarity at protein level between mouse, human and *Xenopus* TAK1 and TAB1 ranges from 90% to 95%. Also for TAB2 and TAB3, the conservation between mouse and human species ranges from 90 to 95%. This is of interest as TAK1 and TAK1 binding proteins have been implicated to function in a number of conserved signaling pathways, including BMP, Wnt and IL-1/Toll signaling[10,12,30,34-36]. Especially the IL-1/Toll signaling pathway, which plays a central role in the innate immune system, is highly conserved from *Drosophila* to mammals[3,33].

Of interest is the high similarity at protein level between TAB2 and TAB3. The overall similarity between these proteins is 46%, but at the N-and C-terminus of the proteins the similarity is approximately 80%. As both proteins contain identical protein domains, they might be performing similar or overlapping functions. With respect to the CUE domain in both proteins, it has been recently shown that the IL-1 signaling protein Tollip also contains a CUE domain and that these domains are required for intramolecular mono-ubiquitination[26,32]. In contrast to poly-ubiquitination of proteins that results in

degradation via the 26S proteosome pathway, protein modulation by mono-ubiquitination has been suggested to serve as a regulatory signal that can alter activity and localization of proteins[26,32]. Moreover, it was shown that TAK1 kinase activity is dependent on ubiquitination and these studies also revealed that TAB2 played an important role in the ubiquitin-dependent phosphorylation of IκBα by TAK1, although it is unclear what the exact role of TAB2 is in this process[37]. Whether the CUE domain of TAB2 and TAB3 is truly involved in localization changes and or altered protein activity remains to be investigated.

Expression of TAK1 and TAB genes

Although TAK1 has been subject of several expression analysis studies, expression of the *Tab* genes has to date not been studied extensively. To examine the expression pattern of *Tab2* and compare it to *Tab1*, *Tak1*, and especially to the highly homologous *Tab3*, we performed RT-PCR and Northern blot analysis. By Northern blot analysis the expression pattern of *Tab2* and *Tab3* in adult mouse tissues was similar. However, clear differences in *Tab2* and *Tab3* transcript expression were observed when several cell lines were tested. This might indicate that *in vivo Tab2* and *Tab3* are not expressed in the same cells within a tissue. Moreover, our data indicate that *Tab3* is expressed at lower levels or in a smaller amount of cells as compared to *Tab2* expression in the cells and tissues examined.

To examine TAB2 protein expression in adult mouse tissues we performed Western blot analysis. TAB2 protein expression can be detected in all tissues examined. Highest TAB2 expression was observed in brain, liver, kidney, heart and lung and lower levels were observed in the hematopoietic tissues, thymus, spleen and BM. The ubiquitous TAB2 protein expression is consistent with the results from the RT-PCR and Northern analysis. Interestingly, TAB2 has been proposed to function in the IL-1 signaling cascade and the TAB2 protein expression pattern is similar to that previously reported for the IL-1 receptor 1 and 2 (IL-1RI and II). High expression of IL-1RI and II has been observed in non-hematopoietic tissues in the adult mouse, including heart, skin and brain and low expression in lymphoid tissues, such as spleen and thymus[4].

Besides the expected 75kD protein band, we also observed a smaller 55-60 kD protein band by Western blot analysis with the antibody directed against the N-terminus of TAB2. The ~50 kD band detected with the antibody directed against the C-terminus of TAB2 is most likely an aspecific background band. As a single band was detected with Northern blotting we ruled out the possibility that two different *Tab2* mRNA splice isoforms would give rise to two different TAB2 protein isoforms. However, recently for human *TAB2* two mRNA variants (accession numbers NM_015093 and NM_145342) have been identified. One *TAB2* mRNA variant contains a translation stop codon within the *TAB2* coding sequence, resulting in a C-terminally truncated TAB2 protein of approximately 60kD. As both human *TAB2* mRNA variants are similar is size, no distinction between these variants could be made by Northern blotting. In a similar fashion mouse TAB2 could also be encoded by two different transcript variants, of which one gives rise to the full length 76 kD protein and the other to the 60 kD protein. Interestingly, the antibody directed against the N-terminus of TAB2 recognized the 55-60kD band with a higher intensity than the ~75kD band in the hematopoietic tissues as compared to the non-hematopoietic tissues. It is currently unclear whether the truncated TAB2 protein has a

similar physiological function as the full length TAB2 protein and further biochemical studies are required to determine the characteristics of this truncated protein. Furthermore, protein sequencing should be performed to conclusively determine the nature of these bands as detected by Western blotting.

TAB2 localization in adult mouse tissues

To more specifically determine the localization of TAB2 we performed immunohistochemistry. Both TAB2 antibodies gave similar results and we were able to detect TAB2 expression in brain, thymus, spleen, lung and bone marrow cells. Interestingly, both TAK1 and TAB2 expression was reported previously in the central nervous system and neuronal cells respectively[1,14]. Similar to the reported expression in neuronal cells[1], TAB2 expression in the adult mouse brain appeared to be localized to the nucleus of the cells. Since the *in vivo* localization of TAB2 is similar to that in neuronal cells, this could indicate that the proposed role for TAB2 in the regulation of NFκB target genes in neuronal cells[1] represents a true physiological function. Besides TAB2 expression in the adult brain, we also observed TAB2 expression in the spinal cord and dorsal root ganglia in the midgestation (E11.5-E12.5) mouse embryo, suggesting a role for TAB2 in development and/or function of the nervous system. Moreover, expression of IL-1 signaling has been reported previously in the brain and was suggested to regulate neurotransmission and to be involved in regulating fever responses [4,5].

In the hematopoietic tissues, thymus and spleen, we were able to detect TAB2 expression in a small number of cells, including the endothelium of some of the blood vessels and few scattered cells throughout the tissue. In the thymus, TAB2 expressing cells were localized in both the medulla and the cortex. In the spleen few scattered TAB2$^-$ expressing cells near blood vessels and at the border (marginal zone) of the red and white pulp area. The marginal zone consists of a continuous ring of marginal zone macrophages[19,20]. Although, the TAB2 expressing cells did not form a continuous ring around the white pulp area, these TAB2 expressing cells could represent a subpopulation of the marginal zone macrophages. Additional immunostainings for TAB2 in combination with markers for macrophages and/or related cell types need to be performed to reveal whether indeed these TAB2 expressing cells are (a subpopulation of) macrophages.

Expression of both TAB1 and TAK1 has been observed in the lung epithelium in midgestation mouse embryos[14,18]. In our studies, we observed low levels of TAB2 expression in lung epithelium in the adult and in midgestation embryos. A higher level of TAB2 expression was detected in the subepithelial layer of the lung in midgestation embryos. Previously, Healy et al. showed that VEGF is expressed in the subepithelial layer of the developing lung and that VEGF could stimulate neovascularization of the lung[7]. Interestingly, vascular endothelium cells and HSCs express the receptor (Flk-1) for the VEGF ligand[22,29,40]. Whether TAB2 expression in cell types known to express either the ligand VEGF or the receptor Flk-1 has any physiological relevance will need to be further investigated. Moreover, the interplay between TAB2, TAK1 and TAB1 in the lung (and in other tissues) awaits further study.

Interestingly, Schmidt-Ullrich et al. reported NFκB activity in several tissues and organs in the embryonic and adult stages of mouse development (as analyzed in a transgenic mouse model with a *Nfκb* promoter driving *lacZ* expression)[28]. These studies revealed NFκB activity from E12.5 onwards in the spinal cord and other neural tissues, and

in the adult mouse brain in a scattered pattern in the outer layers of the cerebellum. This is similar to what we observed for TAB2, although we detected TAB2 expression slightly earlier (E11.5) than the reported NFκB activity. Moreover, in the adult spleen, few scattered cells were found to be lacZ positive, while in the thymus more lacZ positive cells, including lymphocytes could be found. Also lacZ staining was observed in the endothelium of blood vessels[28]. Therefore, NFκB activity in both adult and embryonic stages seems to correspond to the expression pattern of TAB2 we observed in several tissues. This is of great interest as it has recently been shown that TAB2 in neuronal cells is a modulator of NFκB-mediated gene regulation[1]. The similarities in the expression pattern of TAB2 and the reported NFκB activity, suggest that TAB2 not only in neuronal cells, but also in other cell types, is involved in modulating NFκB gene regulation.

TAB2 is localized in the nucleus and cytoplasm of bone marrow cells

As expected from the results of our RT-PCR analysis, we were able to detect TAB2 expression in both the lineage marker negative and the positive fraction of the adult bone marrow. Moreover, we found that TAB2 localized in both the nucleus as well as the cytoplasm in some cells, while in other cells TAB2 localization was restricted to either the nucleus or the cytoplasmic region. TAB2 has been proposed to localize to the nucleus and translocate to the cytoplasm upon IL-1 stimulation[1]. However, as the bone marrow cells are likely exposed to endogenous IL-1 in the bone marrow and moreover were processed during the sorting period in the presence of fetal calf serum (known to contain IL-1), it was not surprising to find TAB2 in the cytoplasm of these cells. Within the bone marrow fraction analyzed, we observed high TAB2 expression in immature myeloid cells and lower TAB2 expression levels in more mature myeloid cells. Also, in some blast-like progenitor cells, we observed high TAB2 expression in the nucleus. Obviously, these findings of TAB2 expression in different subsets of hematopoietic cells need to be extended by co-stainings with hematopoietic lineage markers. Nevertheless, the expression of TAB2 in the myeloid lineage correlates with the finding that IL-1 affects myeloid cells (including monocytes and neutrophils) *in vivo* [2,5,8,15] and with the observed high level of TAB2 transcript expression in the myeloid cell lines (32D and DA3). Moreover, in 32D cells differentiated with the cytokine G-SCF we observed downregulation of *Tab2* RNA expression. In contrast, when 32D cells were differentiated with GM-CSF or when the myeloid precursor cell line MPRO was differentiated with retinoic acid no such *Tab2* expression down-regulation was observed (C.O.; unpublished data). Therefore, these interesting findings of TAB2 expression in the myeloid lineage of bone marrow hematopoietic cells and the possible differential regulation of *Tab2* along the myeloid differentiation pathway, could serve as a starting point for further examination of TAB2 in this hematopoietic cell type.

In conclusion, *Tab2* and *Tab3* gene expression is similar in several adult tissues, which are heterogeneous populations of cells. In contrast, little overlap in expression was observed in the several clonal cell lines analyzed. At protein level TAB2 is ubiquitously expressed in all adult mouse tissues tested. Notwithstanding, when we examined the spatial expression pattern of TAB2 by immunohistochemistry, we were able to detect specific expression in subsets of neuronal cells in the brain, vascular endothelial cells in several adult and embryonic tissues and few scattered cells in the spleen and thymus. Moreover, in the bone marrow, different hematopoietic cell types, most notably the myeloid lineage,

expressed TAB2. Since the observed expression pattern of TAB2 resembles that of reported NFκB activity in the adult mouse, this might indicate that the function of TAB2 is related to NFκB activity.

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Chapter 4

Interleukin-1 signaling in the aorta-gonad-mesonephros region: novel insights into hematopoietic stem cell regulation in the midgestation mouse embryo.

Claudia Orelio, Marian Peeters, Karin van der Horn, Elaine Dzierzak

Abstract

Definitive hematopoietic stem cells (HSCs) are first detectable during mouse development in the aorta-gonad-mesonephros (AGM) region from embryonic day 10.5 (E10.5) onwards. Although several studies have investigated the emergence and regulation of HSCs in the AGM region, our understanding of the regulatory processes underlying their generation is limited. Recently, we reported the upregulation of TAB2, a component of the interleukin-1 (IL-1) signaling pathway, in the AGM region at the developmental time when the first HSCs are detected. IL-1 is a known regulator of bone marrow HSCs. However, the role of IL-1 in regulating HSCs during development has not been investigated. Here we address the role of IL-1 in the regulation of HSCs during midgestation development. We show that the expression of several components of the IL-1 signaling pathway are initiated/upregulated in the AGM region between E10 and E11 and provide evidence that IL-1 signaling is functioning in the E11/12 AGM and liver. The IL-1RI is expressed in/near the endothelium of the dorsal aorta from E11 onwards and is found within c-kit, Mac-1 or CD45 expressing cell populations in the AGM and liver. Moreover, IL-1 has an effect on HSC activity, as AGM and liver explants cultured in the presence of IL-1 show increased HSC activity by *in vivo* repopulation assays. Taken together, these data provide evidence that IL-1 regulates the fate of HSCs from the earliest stages of development onwards.

Introduction

In the adult mouse, hematopoietic stem cells (HSCs) are located in the bone marrow and form the basis of the hematopoietic hierarchy. HSCs are characterized by their ability to self-renew and to give rise to mature hematopoietic cells of all lineages throughout the entire life-span[31,50]. During mouse development, the first primitive hematopoietic cells can be found in the yolk sac from embryonic day 7.5 (E7.5) onwards. These primitive erythrocytes develop in close association with the vasculature of the yolk sac, in so-called blood islands[44]. From E9 onwards multipotent hematopoietic progenitors, with the ability to repopulate the hematopoietic system of newborn recipients and RAG2γc deficient adults, but not that of adult lethally irradiated recipients, can be found in both the yolk sac and within the embryo proper[4,57]. Such multipotent progenitors are first generated in the intra-embryonic para-aortic splanchnopleura[4]. The first definitive hematopoietic stem cells, with the ability to repopulate adult recipients, become detectable and are autonomously generated at E10.5 within the embryo proper in a region referred to as the aorta-gonad-mesonephros (AGM) region[34,37]. This region consists of the dorsal aorta surrounded by mesonephros and the gonads. Closer examination of the AGM region revealed that the first definitive HSCs are localized in the area around the dorsal aorta [6,34,37]. Within the dorsal aorta, clusters of cells that express several hematopoietic (stem) cell markers, have been observed at the ventral aspect of the aorta and these clusters are believed to contain HSCs [27,40,41,53]. From E11 onwards, definitive HSCs become also detectable in the circulation, the yolk sac and the fetal liver[30,34,37]. From E12 until the end of gestation the fetal liver is the main hematopoietic organ and HSC numbers increase dramatically in the fetal liver up to E14 [13,30].

Research has focused on the factors that play a role in the regulation of HSCs during development and although several factors have been proposed to play a role, the understanding of the processes taking place is still limited. In our search for regulators of HSCs we recently reported that the novel gene *Tab2* is upregulated between E10 and E11 in

the AGM region. Moreover TAB2 protein expression was observed in/near the E11 endothelium of the dorsal aorta within the AGM region, the site where HSCs are located [6,34,37,43]. 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 RANK signaling [35,51]. TAB2 has been proposed to function as an adapter protein, involved in binding TAK1 to TRAF6 and bringing the signaling proteins in close proximity of the IL-1 receptor complex, resulting in NFκB and JNK activation [51,52]. In addition, in neuronal cells TAB2 is found in a complex with NFκB at the promoter of several IL-1 responsive genes[1].

The pro-inflammatory cytokine interleukin-1 (IL-1) has been studied intensely for its role in normal physiology and disease. Depending on the target tissue, the cellular context and the local concentration, IL-1 can exert several different biological effects, of which induction of fever and mediating inflammatory responses are the best known[7]. However, IL-1 has also implicated as a regulator of adult mouse and human bone marrow hematopoietic stem and progenitor cells. Expression of IL-1 and the IL-1 receptor has been observed, both at the RNA level and at the protein level, in adult bone marrow populations enriched for HSCs and progenitor cells[25,33,55,56]. Furthermore, functional studies show several IL-1 mediated effects on HSCs and progenitor cells, including radio-protection, growth and/or differentiation and changes in cell adhesion and migration.

The radio-protective effects of IL-1 have been shown both *in vitro* on cultured bone marrow cells as well as *in vivo* upon administration to mice. IL-1 addition prior to irradiation resulted in enhanced long-term hematopoietic recovery [38,42,59]. It has been proposed that contributors to this effect include upregulation of the anti-oxidant enzyme Manganese Superoxidase Dismutase (MnSOD) as well as IL-1 induced G1 phase cell cycle arrest which correlated with increased resistance to cytotoxicity[2,11]. Moreover, IL-1 has been shown to increase the production of other cytokines, including IL-6, GM-CSF and stem cell factor (SCF or kit ligand), which could play a role in the accelerated hematopoietic recovery upon irradiation (reviewed in 7). The finding that blocking antibodies against the receptor c-kit or its ligand SCF, IL-6 or IL-1 itself could diminish the radio-protective effect of IL-1 underlines the importance of the cooperation of IL-1 with other cytokines in exerting its effects[39,42]. Besides increasing the expression of several cytokines, it was shown that IL-1 in combination with other hematopoietic growth factors could induce expansion of hematopoietic progenitor and myeloid precursor cells [3,16,21]. However, other studies report that IL-1 induces differentiation rather than expansion of hematopoietic progenitor cells or that IL-1 completely abrogates HSC activity [26,58]. As these studies were performed with different populations of bone marrow cells, different amounts and combinations of (hematopoietic) growth factors, it is difficult to compare these results. Nevertheless, the fact that in some studies IL-1 has a positive effect on the proliferation of hematopoietic stem/ progenitor cells together with the reported expression of IL-1 and the IL-1 receptor in hematopoietic stem/ progenitors cells[25,32,33] suggests that IL-1 is a positive regulator of adult HSCs and hematopoietic progenitor cells under certain circumstances.

Despite the intense investigations regarding the role of IL-1 as a regulator of HSCs in the adult, very little is known about the regulatory role of IL-1 during development. Since expression of the IL-1 signaling component 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 midgestation embryo. Here we show that several

components of the IL-1 signaling pathway are expressed and that IL-1 signaling is functioning in AGM and liver from E11 onwards. More detailed expression analysis of the IL-1RI revealed expression in/ near the endothelium of the E11 and E12 dorsal aorta and is co-expressed on c-kit$^+$, Mac-1$^+$ and CD45$^+$ AGM and liver hematopoietic cells. Furthermore, long-term transplantation studies reveal IL-1 mediated increases in HSC activity in the AGM region and fetal liver upon organ culture. To our knowledge this is the first study to address the role of the cytokine IL-1 as a regulator of HSCs in the midgestation 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 (CBAxC57/BL10) F1 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 AGM regions and livers were dissected as described [6,37]. For obtaining single cell suspensions of embryonic material, tissues were collagenase treated (0.125% collagenase in PBS/ 10% FCS) for 1 hour at 37°C.

3T3 fibroblasts were cultured in DMEM/ 10% FCS 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. The next day cells were stimulated with IL-1β for target gene induction and IκB degradation studies.

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 min 92°C, 27-40x (40 sec 92°C, 40 sec 58°C, 1-2 min 72°C), 7 min 72°C. PCR primers are listed in 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 EtBr and scanned on a Typhoon scanner (Molecular Dynamics) and analyzed 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 (Tebu Bio) for indicated times. Cells were scraped from tissue culture plates in cold PBS, pelleted 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 hr 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, E12 liver cells and 3T3 cells were stimulated with 10ng/ml and 100ng/ml IL-1β respectively for 0-30 min. Protein lysates were made with RIPA buffer, 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 signaling Technology) followed by α-Rabbit-HRP (DAKO) and visualized with ECL detection.

Organ 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 organ cultures were performed as described previously [34] in the presence of 0, 1 or 10 ng/ml IL-1β (TebuBio). 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 (CBAxC57BL/10)F1 recipient mice. For the expression analysis, cultured tissues were homogenized in TRIZOL 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 [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 and assayed for donor contribution by PCR.

Table 1. RT-PCR primer sequences

Primer name	Primer Sequence (5'-3')	product size	accession number
β-actin for	CCTGAACCCTAAGGCCAACCG	397	X03672
β-actin rev	GCTCATAGCTCTTCTCCAGGG		
Bcl-2 for	GCACAGATGTCCAGTCAGCTG	268	NM_009741
Bcl-2 rev	GCCATATAGTTCCACAAAGGC		
IL-1RacP for	GAAATAGCCTCAGCTCACACAG	458	NM008364
IL-1RacP rev	CTCTGTTCCACCTCAGACTC		
IL-1RI for	ACCTCACTTCTCCTGGATCC	300	NM008362
IL-1RI rev	GGGGTACAAAGAACAAGGCG		
IL-1RII for	CCAGCATCATTGGGGTCAAG	435	NM010555
IL-1RII rev	CCTGGTTGTCAGTCCGTAGC		
IL-6 for	GACTTCACAGAGGATACCAC	442	NM_031168
IL-6 rev	CTCCAGCTTATCTGTTAGGAG		
IRAK4 for	CATGACCAGCCGAATCGTGG	282	AF445803
IRAK4 rev	CAGACACTGGCTAGCAGCAG		
JunB for	GCAGCTCACACACTGGACTC	296	BC003790
JunB rev	CTTTAGACACGAAGTGCGTG		
MMP9 for	ATGTGTCCCACTATACCTCC	464	X72794
MMP9 rev	GACCCAACTTATCCAGACTC		
MnSOD for	CATCCACTTCGAGCAGAAGG	442	X06683
MnSOD rev	AGTCTGAGACTTCAGACCAC		
SCF for	CTTTGGTGAACTTTCATGTG	1042	NM_013598
SCF rev	TGTGGATCACTCCTAAGCCC		
TAB1-910 for	CAGGCCAACCAGGATTGC	454	NM_025609
TAB1-1360 rev	GAGCTGGAGCTGCTGCTCTG		
TAB2-1680 for	TGCTGGTGCACCAGAAGGCCAGGATG	686	BC004813
TAB2-2350 rev	GTCTCTCAGGCCTTTCAGGTG		
TAK1-3'UTR for	GTGGTACATTACAGAGGGAC	412	BC006665
TAK1-3'UTR rev	CCGATAGCTCAGCTCAAGCC		
TRAF6 for	CATTTATGCACCTGGAAGCC	423	NM_009424
TRAF6 rev	CCCATGGAAGCACAGTGAAG		

FACS analysis

Single cell suspensions were stained with IL-1RI (BD biosciences) antibody followed by α-rat-biotin (BD biosciences), streptavidin-PE (Caltag) antibodies on ice for 30 minutes. Cells were co-stained with FITC labeled antibodies for c-kit, MAC-1 or CD45 (Pharmingen). Dead cells were excluded by 7AAD (Molecular Probes) and FACS analysis was performed on FACScan (Becton Dickinson).

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 (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).

Results

IL-1 signaling components are expressed in the midgestation AGM and liver.

Recently, we reported upregulated expression of mTAB2 in the mouse AGM region, at the time when the first adult repopulating HSCs are being generated[43]. As TAB2 is the binding partner of TAK1 in the IL-1 receptor signal transduction pathway[51,52], we examined whether other components of the IL-1 signaling pathway are expressed in the midgestation AGM region and liver. Therefore, RT-PCR expression analysis was performed for the IL-1 receptors I and II, the IL-1 co-receptor IL-1RAcP (receptor associating protein) and for IL-1 signaling mediators (TAB1, TAB2, TAK1, TRAF6 and IRAK-4) on E10 AGM and E11 and E12 AGM and liver cDNAs.

As shown in Figure 1, the transcription of *Tab2* and *Tak1* is initiated between E10 and E11 in the AGM region. *Tab1* is expressed in the E10 AGM (data not shown) and thereafter all 3 genes are expressed in the E11 and E12 AGM and liver. IL-1 receptor signaling is mediated via the IL-1RI and the IL-1RAcP co-receptor. Expression of both *Il-1 receptor* genes was observed in the E11 and E12 AGM and liver. Moreover, the expression of *Il-1β* and the non-signaling *Il-1rII* can be detected from E11 onwards in the AGM and liver. No expression of *Il-1β*, *Il-1rI* and *Il-rII* could be detected in the E10 AGM. Thus, *Il-1rI, Il-1rII* and *Il-1β* transcription is initiated between E10 and E11. In contrast, initiation and upregulation of the *Il-1racp* occurs one day earlier (at E10). Also, the essential signaling mediators of the IL-1 signaling cascade, *Traf6* and *Irak-4*, are highly expressed from E10 onwards in both AGM and fetal liver tissue.

These data show at transcriptional level that several components essential for IL-1 receptor signaling are initiated and expressed at E10 and/or thereafter in the AGM region. Interestingly, this temporal expression pattern of the *Il-1 receptors* parallels the emergence of HSC activity in this region.

IL-1 signaling is functional in the AGM and fetal liver from E11 onwards

To determine whether the IL-1 signaling pathway functions in the midgestation AGM region and liver, we tested for the activation of IL-1 target genes in *in vitro* cultures. IL-1 receptor signaling can be measured by NFκB or JNK activation in target cells upon IL-1 stimulation (via reporter assays, kinase assays or via IκB degradation)[9] or by regulation of known IL-1 target genes, such as *JunB* , *Il-6* or *Mnsod*[7,12].

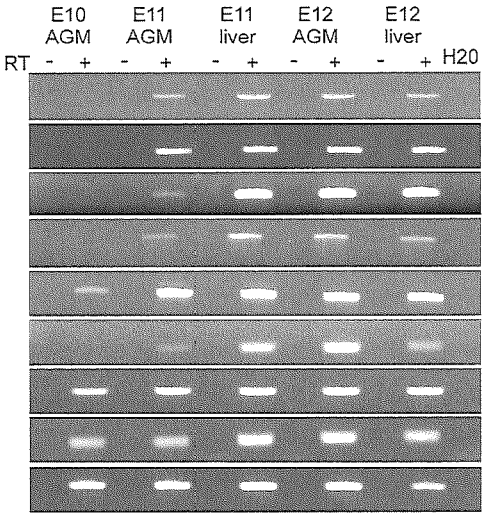


Figure 1. Expression analysis of IL-1 signaling components in the AGM region and liver.

RT-PCR analysis was performed to determine the expression of the IL-1 receptors I and II (IL-1RI and IL-1RII), the IL-1 accessory receptor (IL-1RAcP) and several downstream signaling components, including TAK-1, TAB2, TRAF6 and IRAK-4 in the midgestation AGM region fetal liver. RNA was isolated from E10-E12 AGM and liver and used for cDNA synthesis and RT-PCR under standard conditions. For each primer set optimal conditions were determined and PCR was performed for 30-40 cycles. Besides the differential expression of TAB2, also TAK1, IL-1RI, IL-1RII, IL-1RAcP and IL-1β are differentially expressed between E10 and E11 in the AGM region. Moreover, the downstream signaling components IRAK-4 and TRAF6 are expressed from E10 onwards in the AGM and liver. RT=reverse transcriptase

Conditions for the transcriptional induction of *JunB* by IL-1 were tested first in embryonic 3T3 fibroblasts. Cells were either left untreated or stimulated with 10ng/ml IL-1β for 30 minutes, 1 hour or 2 hours. Subsequently RNA was isolated and used for cDNA synthesis. Semi-quantitative RT-PCR for *JunB* (Figure 2A) was normalized against *Actin* expression levels to calculate the fold induction of *JunB* gene expression by IL-1β (Figure 2B). This analysis revealed that *JunB* levels increased upon IL-1 stimulation peaking at 1 hour with a 4.5 fold higher expression level as compared to untreated cells. Furthermore, 3T3 cells were stimulated with (10pg/ml) IL-1β in the presence of cyclohexamide or an antibody that neutralizes IL-1β activity (Figure 2C). Since *JunB* is an immediate early target gene of IL-1 signaling, inhibition of protein synthesis by cyclohexamide should not result in a decrease of *JunB* induction by IL-1. As expected IL-1 induced *JunB* expression is not reduced in the presence of cyclohexamide and the fold induction is similar to that found in the previous experiment (3.5 fold versus 4.5 fold Fig.2B). More importantly, the IL-1 blocking antibody is able to reduce *JunB* expression levels similar to that found in untreated cells, confirming that the increase of *JunB* expression is indeed specifically induced by IL-1β. Hence, this RT-PCR analysis can be used to specifically measure IL-1β target gene induction in cells.

E10, E11 and E12 AGM and liver tissues were dissected and cells were cultured overnight prior to IL-1β stimulation. Increased *JunB* expression (range 2.5-60 fold; n=5) was detected in E11 and E12 AGM and liver cells treated with IL-1β for 30 min up to 2 hours as compared to untreated cells (Figure 2D/E). Additionally, IL-1β induced upregulation of other target genes, including *Mnsod* and *Il-6*, was observed (Fig. 2D/ E and data not shown). In order to more specifically quantify the results from the RT-PCR

analysis we performed cDNA dot blot analysis. Similar results were obtained (data not shown). Treatment of E11 AGM cells with cyclohexamide prior to IL-1 stimulation did not result in a complete inhibition of IL-1 induced *JunB* expression (Figure 2F). Consistent with our RT-PCR expression analysis in which we did not observe expression of the IL-1 receptor in E10 AGM cells, we were unable to detect induction of junB expression upon IL-1 stimulation in these cells (data not shown).

Another well established method to determine IL-1 signaling is based upon IL-1 induced IκB degradation, which is a prerequisite for NFκB activation and signaling. Therefore, we also performed Western blot analysis for IκB on untreated and IL-1 stimulated 3T3 and E12 liver cells. As shown in Figure 2G, IL-1 stimulation results in IκB degradation in both E12 liver and 3T3 cells. The observation that in E12 liver cells moderate levels of IκB protein can still be detected after IL-1 stimulation is likely due to the fact that IκB is expressed in cells that do not express the IL-1R, and therefore IκB in these cells is not targeted for degradation.

Altogether, these experiments show that IL-1β is able to increase the expression of known IL-1 target genes in E11/ E12 AGM and liver cells and these results correlate with the detected expression of the IL-1 receptor and its signaling components within these tissues. Moreover, also at the protein level as measured by IκB degradation, IL-1 signaling is functional in E12 liver cells. Together, these data show that a population of cells within the E11/ E12 AGM and fetal liver are IL-1 responsive and contain functional IL-1 signaling properties.

The IL-1RI is expressed on hematopoietic cells in the E11 AGM and liver.

To further investigate whether expression of the IL-1RI coincides with a specific population of cells (i.e. hematopoietic), flow cytometric analysis was performed on AGM and liver cells. Expression of the IL-1RI was examined in combination with CD45, c-kit or Mac-1. CD45 is a pan-hematopoietic marker, while c-kit and Mac-1 have been used to enrich for HSCs in the E11 AGM and liver[46].

As shown in a representative experiment (Figure 3A), expression of the IL-1RI in E11 AGM tissue can be readily detected above background levels (top panels). Moreover, we observed the expression of c-kit, CD45 and Mac-1 on IL-1RI expressing E11 AGM cells (lower panels). Further FACS analyses (summarized in Figure 3B) reveals that in the E11 AGM and liver approximately 0.5-3.7% of the cells are IL-1RI$^+$/CD45$^+$ and that almost all (85%) IL-1R1$^+$ cells are CD45$^+$. FACS analysis for the IL-1R1 in combination with c-kit in the E11 AGM and liver shows that 0.5-5.0% of the cells are IL-1RI$^+$/c-kit$^+$. Approximately half (42-52%) of the IL-1R$^+$ cells in these tissues also expressed c-kit, while only 7-19% the c-kit$^+$ cells also express the IL-1R1. Also 36-38% of the IL-1RI$^+$ AGM and liver cells are Mac-1$^+$ and in the liver more Mac-1$^+$ cells are IL-1RI+ (62%) than in the AGM region (33%). Thus, there appears to be great overlap in the expression of HSC markers and the IL-1RI in the cells of E11 tissues that harbor HSCs, suggesting that HSCs express the IL-1RI. This is further supported by FACS data from subdissected E11 aorta tissue in which 0.6% of the cells are IL-1RI$^+$/c-kit$^+$, 1.1% of the cells are IL-1RI$^+$/CD45$^+$ and 0.9% of the cells are IL-1RI$^+$/Mac-1$^+$ (data not shown).

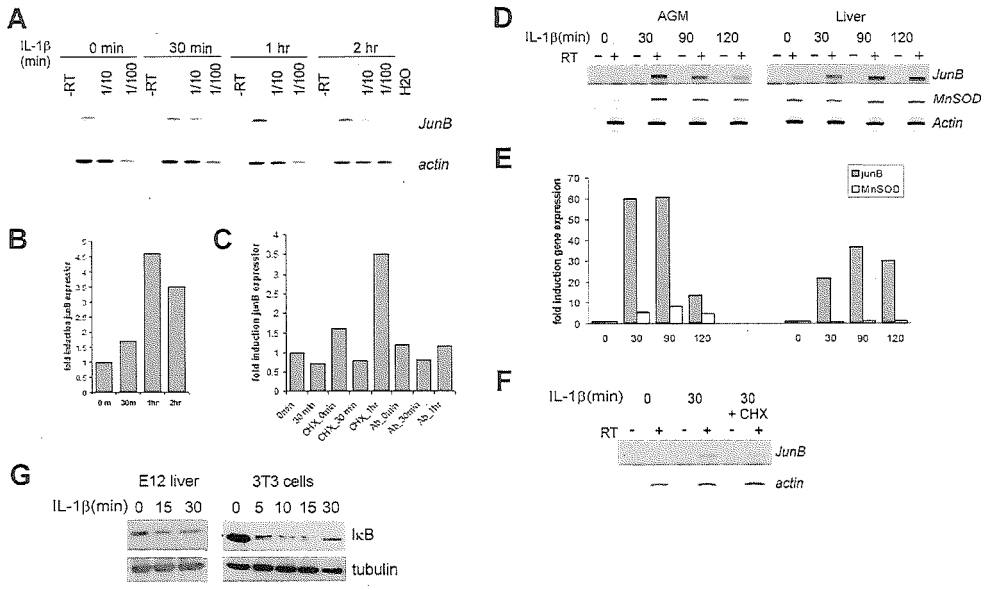
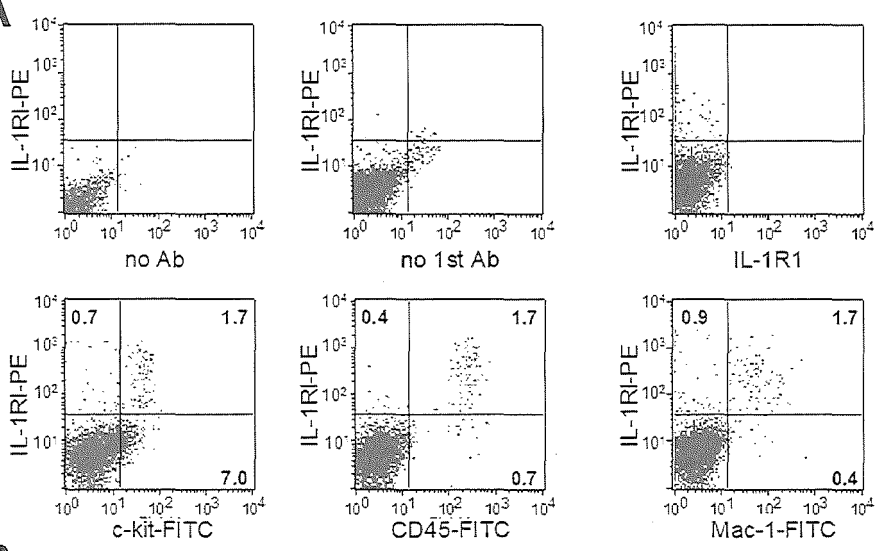


Figure 2. IL-1 signaling in 3T3 fibroblasts and midgestation AGM and liver cells.
(A) 3T3 fibroblasts were stimulated with IL-1β (10 ng/ml) for 0 min, 30 min, 1 or 2 hrs and RNA was isolated and used for cDNA synthesis. Serial dilutions of the cDNA were used for semi-quantative RT-PCR and analysis was 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. (D) Single cell suspensions of E11 AGM and liver cells were treated with IL-1β (10 ng/ml) for 0, 30, 90 or 120 min. RNA was isolated and cDNA generated and used for semi-quantitative RT-PCR analysis. Representative semi-quantative RT-PCR for the IL-1β target genes JunB and MnSOD. In E11 AGM and liver cells a clear induction of JunB expression is observed within 30 min after IL-1β stimulation. For MnSOD only in E11 AGM a clear gene expression induction is observed upon IL-1β stimulation. In E11 liver cells, the IL-1β stimulation does not increase the high basal level of MnSOD gene expression. (E) Fold induction of JunB and MnSOD expression after IL-1β stimulation of AGM and liver cells as calculated from RT-PCR values. (F) IL-1β induced JunB target gene expression in E11 AGM cells is slightly inhibited by cyclohexamide (CHX). (G) IL-1β stimulation of E12 liver cells (left panel) or 3T3 fibroblasts (right panel) results in rapid IκB degradation.

To test whether co-expression of the IL-1R1 with the hematopoietic (stem) cell markers CD45, c-kit and Mac-1 is restricted to hematopoietic cells during development or whether this could also be observed in the adult, we performed similar FACS analysis on adult bone marrow samples (n=3). Again we observed co-expression of the IL-1R1 with $CD45^+$, c-kit^+ and Mac-1^+ cells. In contrast to the E11 AGM and liver where approximately 85% of the IL-1R1+ cells express the CD45, in the bone marrow all IL-$1R^+$ cells express CD45, suggesting that the IL-1R1 in the bone marrow is expressed exclusively on

A



B

	E11 AGM		E11 liver		bone marrow	
	mean	(range)	mean	(range)	mean	(range)
IL-1RI$^+$/c-kit$^-$	0.9	(0.2-1.5)	2.2	(1.0-5.9)	2.7	(2.1-3.9)
IL-1RI$^-$/c-kit$^+$	5.2	(3.8-8.6)	25.4	(13.0-33.1)	7.9	(6.1-10.5)
IL-1RI$^+$/c-kit$^+$	1.2	(0.5-1.8)	2.0	(0.6-5.0)	3.6	(2.1-6.5)
IL-1RI$^+$/CD45$^-$	0.3	(0.2-0.6)	0.5	(0.1-1.0)	0.0	(0.0)
IL-1RI$^-$/CD45$^+$	0.8	(0.5-1.0)	9.4	(7.7-11.2)	81.9	(76.2-85.3)
IL-1RI$^+$/CD45$^+$	1.7	(0.5-2.3)	2.3	(1.6-3.7)	6.8	(3.9-12.3)
IL-1RI$^+$/Mac-1$^-$	0.7	(0.2-1.3)	1.1	(0.9-1.4)	1.8	(1.3-2.9)
IL-1RI$^-$/Mac-1$^+$	0.6	(0.5-0.7)	2.9	(1.8-4.2)	49.6	(47.4-52.2)
IL-1RI$^+$/Mac-1$^+$	1.2	(0.4-1.8)	1.8	(1.2-2.2)	4.6	(2.1-8.6)

Figure 3. Expression of the IL-1RI with c-kit, CD45 and Mac-1 in the E11 AGM and liver and adult bone marrow.

As the IL-1RI is known to be expressed in primary cells with few receptors per cell (as low as 50 receptors per cell [7]) FACS staining for the IL-1RI was performed with intermediate biotin-streptavidin-PE steps to enhance the signal. (A) The representative FACS analysis plots in the top row show the results of E11 AGM cells with no antibody (left), without the IL-1RI antibody (only the secondary antibody and streptavidin-PE; middle) or stained with the IL-1RI antibody (right). In the bottom panel the results for AGM cells stained with IL-1RI in combination with c-kit, CD45 or Mac-1 are shown. The percentages of positive cells are shown in each quadrant. (B) The table summarizes the FACS data (percentages of single and double positive cells) from E11 AGM and liver from 4 independent experiments with 6 litters of embryos. The data for adult bone marrow are derived from 2 independent experiments with 3 adult male mice.

hematopoietic cells. Most interestingly, approximately 30% of the c-kit$^+$ BM cells express the IL-1R1, suggesting that the IL-1RI may indicate a subpopulation of BM HSCs.

Taken together, these data demonstrate that the IL-1RI is expressed mainly on hematopoietic cells and possibly HSCs in the midgestation AGM and liver and, in the adult BM is expressed only on hematopoietic cells.

High IL-1RI expression is localized to the midgestation dorsal aorta.

To more specifically localize IL-1RI expressing cells in the E11 and E12 AGM we performed immunohistochemistry. As shown in Figure 4A, expression of the IL-1RI can be found in/ near the endothelium of the dorsal aorta at E11. Highest expression is found at the ventral aspect of the aorta, the site harboring hematopoietic clusters which are believed to contain HSCs. At E12 the staining for the IL-1RI is more intense at the ventral aspect and can also be found around the entire circumference of the dorsal aorta (Figure 4B). IL-1RI expression is localized in/near the endothelium of the dorsal aorta. Thus, the temporal and spatial expression of the IL-1RI suggests that it may play a role in HSC development.

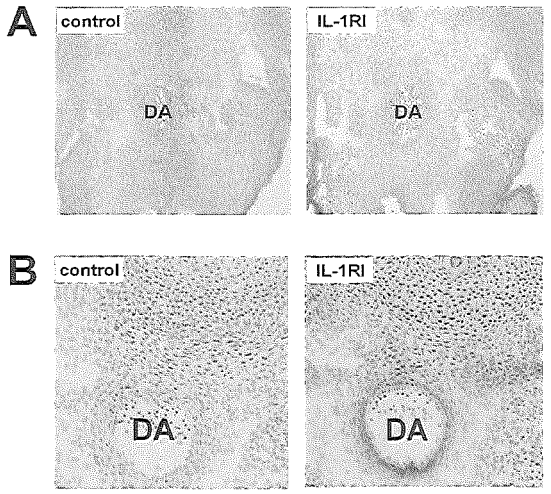


Figure 4. IL-1RI is expressed in the aorta of midgestation AGM region
Transverse cryosections of the trunk region of E11/E12 embryos. The dorsal site is at the top and ventral site is at the bottom of each section. Immunohistochemistry was performed with an IL-1RI antibody. The left panels show the negative control (no IL-1RI antibody) and the right panels show sections stained with the IL-1RI antibody (A) E11 section (original magnification 100x). (B) E12 section (original magnification 200x). In the E11 embryos the staining for the IL-1RI is located mainly at the ventral side of the dorsal aorta and at E12 the staining for the IL-1RI is more intense and located around the dorsal aorta.

IL-1β increases HSC activity in the AGM and fetal liver

As IL-1 signaling is functional in AGM cells and the IL-1RI is expressed in the E11/ E12 dorsal aorta as well as on c-kit expressing cells, we investigated whether IL-1β affects HSC activity in the E11 AGM and liver by explant cultures. The previously described 3 day organ culture system was used, in which HSCs are increased and/or maintained in the AGM and liver tissues[6,30,37]. E11 AGM or liver explants were cultured in the presence or absence of 1 ng/ml or 10 ng/ml IL-1β for three days. After the culture a single cell suspension was obtained and different amounts of cells (0.3-2ee; embryo equivalents) were injected into lethally irradiated recipients (Figure 5A). Four months post-

transplantation, PCR analysis for the donor marker was performed on peripheral blood DNA of the recipients.

As shown in Figure 5B, upon transplantation of 1 ee, the HSC activity in AGM tissue cultured in the presence of a low concentration (1ng/ml) IL-1β is higher as compared to AGM cultured without IL-1β (100% vs. 57% mice). However, a higher concentration (10ng/ml) IL-1β did not clearly affect HSC activity in the AGM (50% mice repopulated) as compared to the tissues cultured in the absence of IL-1β. Similar results were obtained with 0.3ee of transplanted AGM tissue. For cultured E11 liver tissues, regardless of the concentration of IL-1β, the presence of IL-1β during the culture period increased the HSC activity. Multi-lineage analysis was performed on a number of repopulated recipients. In all cases high level repopulation was observed in all hematopoietic tissues tested (data not shown).

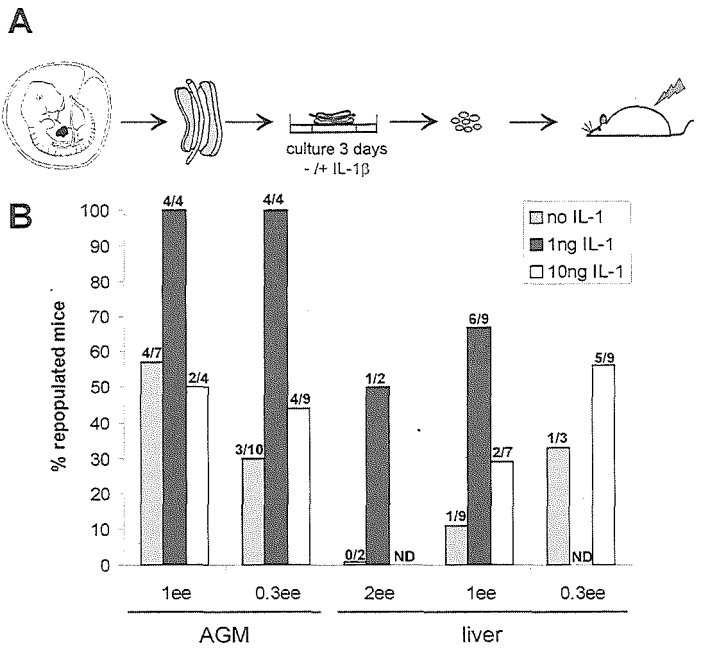


Figure5. IL-1 increases HSC activity in E11 AGM and liver organ cultures.

(A) Schematic overview of the protocol used to examine the effects of IL-1 on HSCs. From E11 embryos (marked with Sca-1-GFP or human β-globin) AGM or liver tissues were dissected and cultured for 3 days on a filter at a liquid-air interface in the presence or absence of IL-1β. After 3 days, single cell suspensions were obtained by collagenase treatment and injected into lethally-irradiated recipients at 0.3-2 embryo equivalents (ee). Four months after the transplantation PCR analysis for the donor marker (GFP or human β-globin) was performed to determine hematopoietic repopulation of the recipients. Only those recipients with greater than 10% donor-derived reconstitution are considered repopulated. (B) Results of the transplantation assay from the organ experiments in the presence of 0, 1 or 10 ng/ml IL-1β. Addition of 1 ng/ml IL-1β to the AGM and liver organ cultures results in increased numbers of repopulated recipients at different doses of injected cells. At the concentration of 10 ng/ml IL-1β no clear effect is observed in the AGM organ cultures. Increased numbers of repopulated recipients are observed with liver tissue cultured with 1 or 10 ng/ml IL-1β as compared to the recipients injected with control liver tissue (0ng/ml IL-1β). Results in this graph are derived from 4 independent transplantation experiments. The bars represent the percentage of repopulated recipients and the numbers above the bars indicate the number of repopulated recipients out of the total number of recipients injected.

Expression analysis of cultured E11 AGM and liver tissues

Based on the results from the transplantation assays showing long term, high level, and multi-lineage engraftment of the recipients, we conclude that IL-1β can increase LTR-HSC activity in both the E11 AGM region and liver upon culturing. Moreover, we show that the effect of IL-1β is concentration-dependent, with lower concentrations having a more potent effect than a higher concentration on the LTR-HSC activity in the E11 AGM and liver.

The effect of IL-1 resulting in increased HSC activity in the cultured AGM and liver tissues could be due to increased HSC proliferation, decreased HSC apoptosis or altered properties of the HSCs or the microenvironment. To gain insight into the processes affected by IL-1 during the culture of AGM and liver tissues, we performed RT-PCR expression analysis on freshly isolated (non-cultured) E11 AGM and liver and tissues cultured either in the presence or absence of IL-1β. Since IL-1 is known to regulate expression of the IL-1 receptors and of IL-1 itself, we first investigated expression of these genes. As shown in Figure 6, expression of the *Il-1rI* and *Il-1racp* remains unchanged during the culture period, either in the presence or absence of IL-1. Moreover the expression level is similar to that of non-cultured AGM or liver tissue. However, the expression of *Il-1* and the non-signaling *Il-1rII* seems to be upregulated in tissues cultured in the presence of IL-1. In addition, the expression level of the IL-1 signaling component, *Tab2* is similar in non-cultured and cultured tissues.

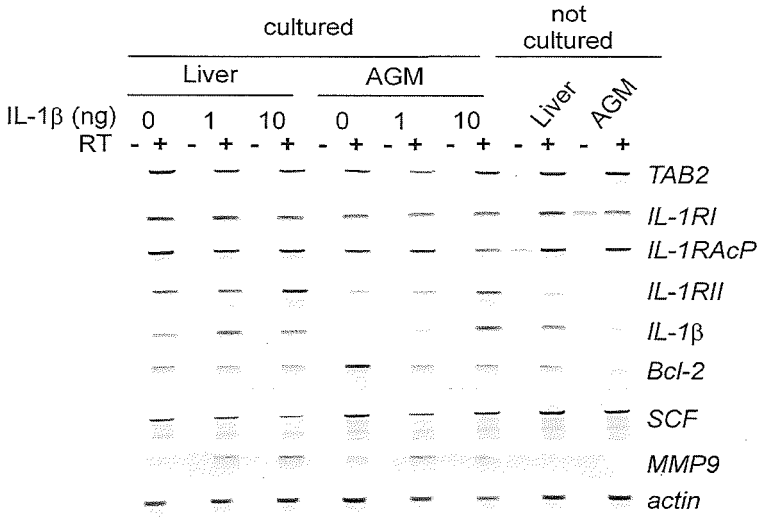


Figure 6. Expression analysis of cultured E11 AGM and liver tissue.
RNA was isolated from fresh, non-cultured E11 AGM and liver tissue or from tissues cultured for three days in the presence of 0, 1 or 10 ng/ml IL-1β in an organ culture system as used for cDNA synthesis. RT-PCR analysis reveals that expression of TAB2, IL-1RI, IL-1RAcP and stem cell factor (SCF) remains relatively constant throughout the culture period as well as compared to non-cultured tissues. Expression of both the decoy IL-1RII and IL-1β appears to slightly increase during the culture period in tissues cultured in the presence of IL-1β. Interestingly, expression of the matrix metalloproteinase-9 (MMP9) is induced in AGM and liver tissue cultured in the presence of IL-1β.

To investigate whether IL-1 could play a role in proliferation or apoptosis of HSCs during the culture period we also examined the expression of the HSC growth factor *Scf* (kit-ligand) and the anti-apoptotic *Bcl-2* gene. The expression of both genes remained at similar levels in the liver cultures in the presence or absence of IL-1 and in comparison to non-cultured liver tissue. However, *Bcl-2* expression appears to increase in AGM explants as compared to non-cultured tissue. For another anti-apoptotic gene, *Bcl-x*, we observed an increase in expression in the liver tissue cultured in the presence of IL-1 as compared to tissue cultured in the absence of IL-1. In cultured AGM tissue *Bcl-x* expression was unchanged in the presence of IL-1 (data not shown). Most interestingly, the *Matrix metalloproteinase-9* (*Mmp-9*) gene was upregulated dramatically when AGM and liver tissue was cultured in the presence of IL-1β. Previously, MMP-9 has been implicated in both IL-1 and SCF processing[22,24,48] and thus the expression changes observed after the culture of AGM and liver with IL-1 suggest a role for IL-1 signaling in hematopoietic and/ or HSC regulation.

The role of TAB2 in IL-1 induced effects.

The previously reported TAB2 expression in the AGM region[43] and the IL-1RI expression as reported here, show that TAB2 is expressed in a temporal and spatial manner similar to the IL-1RI. Since it was been reported that in neuronal cells TAB2 is translocated from the nucleus to the cytoplasm upon IL-1 stimulation[1], we investigated whether this IL-1 induced translocation of TAB2 could also take place in hematopoietic cells.

Therefore we tested a hematopoietic progenitor cell line 416B and control 3T3 fibroblast cells for their ability to translocate TAB2 upon IL-1 stimulation. The 416B hematopoietic progenitor cell line was chosen for this study because of the technical limitions imposed by the scarcity of AGM and fetal liver HSCs. In untreated 416B and 3T3 cells we observed highest levels of TAB2 in the nucleus and lower levels in the cytoplasm. Upon IL-1 stimulation, we were unable to observe TAB2 translocation from the nucleus to the cytoplasm in either cell line. Thus, IL-1 induced TAB2 translocation may be specific to neuronal cells. Alternatively, the cell lines used for these experiments may be transformed or may lack some (functional) component required for TAB2 translocation. Further investigations are required to reveal how TAB2 is involved in IL-1 signaling in hematopoietic cells.

Discussion

Components of IL-1 signaling pathway are expressed and functioning in the midgestation AGM and liver

Hematopoiesis during development is a highly dynamic process and several factors are believed to be involved in the emergence and regulation of hematopoietic cells. Although several growth and differentiation factors have been identified to play a role in the induction and regulation of HSCs in the embryo, there are still gaps in our understanding of the processes that take place. Especially little is known concerning the role that cytokines play during development, while their role in adult hematopoiesis has been delineated to a great extent. Here we report that several components of the IL-1 signaling cascade, including the cytokine *Il-1*, the receptors *Il-1rI, II* and *Il-1racp* as well as several signaling mediators are differentially expressed between E10 and E11 in the AGM region. Appearance of their expression coincides with the emergence of HSC activity in

this region. Besides expression of these genes we also show that IL-1 signaling is functional by means of induction of gene expression of known IL-1 target genes and by IκB degradation.

A large number of IL-1 target genes are known (reviewed in 7) and these include cytokines and their receptors, adhesion molecules, acute phase reactants, pro-inflammatory mediators and transcription factors. TAK1, the binding partner of TAB2, was shown previously to mediate the IL-1 induced *JunB* gene expression[12]. Since both TAK1 and the IL-1 receptor are differentially expressed from E11 onwards in the AGM region, *JunB* was one of the most obvious target genes for our investigations. Indeed, we found potent *JunB* induction upon IL-1 stimulation in E11 AGM and liver cells. However, *JunB* expression can also be induced upon cellular stress. Therefore, we confirmed the specificity of the IL-1 mediated *JunB* gene expression induction with an IL-1 blocking antibody. In 3T3 cells the induction of *JunB* expression by IL-1 is similar in the presence or absence of the IL-1 blocking antibody, showing that *JunB* induction is mediated via IL-1 and is not due to cellular stress. Moreover, we also observed *Mnsod* induction, another target gene downstream of IL-1 induced by NFκB signaling. Thus several downstream mediators of IL-1 signaling are functioning in the midgestation AGM and liver.

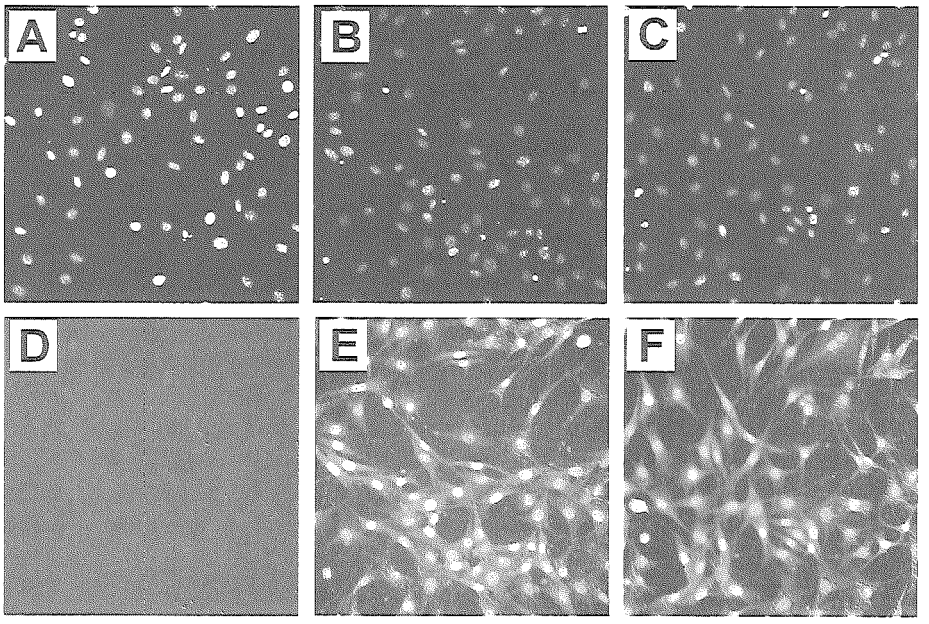


Figure 7. TAB2 intracellular localization in 3T3 fibroblasts
3T3 cells were cultured on gelatin coated coverslips, serum-starved and subsequently stimulated with 100ng/ml IL-1β for 0 min and 2 hours. Cells were fixed and processed for TAB2 immunofluoresence staining. (A)(B)(C) nuclear DAPI staining (D)(E)(F) TAB2 staining. (A)(D) negative control for TAB2 staining; (B)(E) untreated 3T3 cells (C)(F) 3T3 cells stimulated for 2 hours with IL-1β. TAB2 is localized in both the nucleus and to a lower extent in the cytoplasm and no change in the intracellular TAB2 localization is observed upon IL-1β treatment of the cells.

To further confirm that IL-1 signaling indeed is functional we also employed Western blotting to detect degradation of IκB in 3T3 and liver cells. Upon IL-1 receptor signaling the canonical NFκB pathway is activated in which IκB, the inhibitor of NFκB, is phosphorylated and targeted for degradation, thereby relieving NFκB and allowing NFκB translocation to the nucleus and further regulation of target genes. As expected, we indeed found a decrease of IκB protein expression rapidly after IL-1 stimulation in E12 liver cells and in 3T3 cells. The fact that IκB protein expression in the E12 liver cells did not decrease as dramatically as seen in the 3T3 cells is likely due to the mixed population of cells within the E12 liver with only a small population of cells expressing the IL-1RI.

Expression of the IL-1RI in midgestation hematopoietic organs and the effect of IL-1 on HSC activity

A closer investigation of protein expression analysis by FACS and immunohistochemistry revealed that the IL-1RI in the E11 AGM is expressed in/near the endothelium of the dorsal aorta, with highest expression in the ventral aspect. However, in the E12 AGM, the IL-1RI expression is not restricted to the ventral aspect, but expression is observed in/ near the endothelium surrounding the entire aorta. Furthermore, FACS analysis revealed that the IL-1RI is expressed on 19% of the c-kit$^+$ cells, which marks the HSCs in the AGM. Also in the E12 AGM and E11/E12 liver we found IL-1R$^+$/c-kit$^+$ cells. Together these results indicate that IL-1 might be directly regulating HSCs. We further investigated this by performing organ cultures in the presence of IL-1β. These studies revealed that HSC activity, as detected by *in vivo* repopulation, in both the AGM and liver are increased upon IL-1β stimulation as compared to non-treated control tissues. Together with the results showing expression of the IL-1RI in a population of cells in the E11 AGM region and liver known to contain HSCs, these functional data indicate that IL-1 might be an *in vivo* regulator acting directly on HSCs. Despite the fact that we observed a clear impact on HSC activity upon addition of IL-1β during organ culture of AGM and liver tissue, previous studies of IL-1 ligand and receptor deficient mice did not reveal a crucial role for IL-1 during development. Mice deficient for *Il-1α*, *Il-1β* or the *Il-1rI* are viable, but display impaired immune function, most particularly within the innate immune system and lymphocyte activation[18,23]. However, it cannot be excluded that other IL-1 related proteins are functionally redundant with IL-1 and mask the effects of IL-1 deficiency[8,29,49]. Moreover, the observation that a lower concentration of IL-1 had a more potent effect on AGM and fetal liver HSC activity than a higher concentration is similar as reported previously for adult BM HSCs [28].

It is currently unclear what the impact of IL-1 on AGM and liver HSCs is. Increased proliferation, decreased apoptosis or altered cellular characteristics of HSCs could be induced by IL-1. Further investigations will be aimed to determine these effects. These studies will include determining the amount of c-kit$^+$ cells after organ culture in the presence or absence of IL-1, as well as BrdU incorporation studies to investigate the amount of proliferation within the HSC population. Moreover, FACS analysis for Annexin or other apoptotic markers (i.e. TUNEL staining) could provide insight into apoptotic processes taking place under the influence of IL-1. Also, expression analysis for a panel of anti-apoptotic and pro-apoptotic genes, such as *Bcl-2*, *Bim* and *Bcl-x*, will be examined to gain insight in the role of IL-1 on apoptosis in the AGM and liver HSC population.

Interestingly, during inflammatory processes 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 chemo-attractant cytokines, such as IL-8, in epithelial and fibroblast cells[7,15,36]. IL-1 has also been implicated in the mobilization of HSCs from the adult BM to the peripheral blood [17] and to enhance the adhesion of $CD34^+$ bone marrow cells to bone marrow vascular endothelial cells [45,54]. For this reason investigating the effects of IL-1 on AGM HSC adhesion and migration would be interesting. Moreover, the expression analysis in organ cultures revealed that *Mmp-9* is upregulated in both the AGM and liver tissues upon organ culture in the presence of IL-1β. It had already been shown by others that IL-1 induces *Mmp-9* expression in vascular cells [10,14,19,20]. Moreover MMP-9 has been implicated in regulating the bio-activity of IL-1 and SCF and in HSC migration within the adult bone marrow[22,24,48]. Therefore *Mmp-9* might be a very interesting IL-1 target gene in the AGM and liver and may be involved in regulating HSC migration from the AGM to the liver and subsequently to the bone marrow. Further investigations should provide insight into the protein expression and functional activity of MMP-9 in the AGM region and liver.

The role of TAB2 in IL-1 signaling

Although the role of TAB2 in IL-1 signaling in the AGM region was not addressed in our present studies, we would like to discuss its potential role here. Our interest in the IL-1 signaling pathway in the AGM region began when we found *Tab2* gene expression upregulated in this tissue at the time of HSC emergence. TAB2 was originally proposed to function as an adapter protein in IL-1 signaling. More recently, it was shown that IL-1 signaling is not impaired in *Tab2* deficient mice[47]. However, *Tab2* deficiency does results in an embryonic lethal phenotype around E12.5 due to fetal liver degeneration. Although the fetal liver is the main hematopoietic site in the embryo at E12.5, it was not investigated whether hematopoiesis or IL-1 signaling in these cells is disturbed in *Tab2* deficient mice[47]. Nonetheless, others have shown that in neuronal cells TAB2 is involved in NFκB activity regulation upon IL-1 stimulation[1], suggesting that TAB2 does play a role in IL-1 signaling, although a different role than originally proposed. As shown in neuronal cells, we also wanted to investigate whether IL-1 could regulate TAB2 translocation from the nucleus to the cytosol. However, we were unable to observe TAB2 translocation upon IL-1 stimulation in 3T3 fibroblast and 416B hematopoietic cells. It is currently unclear why we failed to observe this translocation, although for the 416B cell line we also did not observe IκB degradation upon IL-1 stimulation (data not shown) suggesting that IL-1 signaling might be impaired in this transformed cell line. The fact that in 3T3 cells without IL-1 treatment TAB2 is localized within both the nucleus and the cytosol might have interfered with detecting TAB2 translocation to the cytosol. However, we cannot exclude the possibility that some component required for TAB2 translocation is not functional in 3T3 cells. Based on previous studies by others, it appears that there is a role for TAB2 in IL-1 signaling[1,51] and that TAB2 may play an essential role in the main hematopoietic organ during midgestation development, the fetal liver, and possibly also in the AGM region, but that the specific function of TAB2 is restricted to specific cell types.

In conclusion, this study reveals the differential expression of functional IL-1 signaling components in the AGM coincident with the appearance of HSC activity in this region. We observed that IL-1R expression is detected in cell populations known to contain HSC activity and that IL-1 can increase HSC activity of midgestation AGM and liver cells. Together these data strongly suggests that IL-1 is a regulator of the first detectable adult repopulating HSCs during mouse development.

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Chapter 5

The role of apoptosis in the development of AGM hematopoietic stem cells revealed by Bcl-2 overexpression

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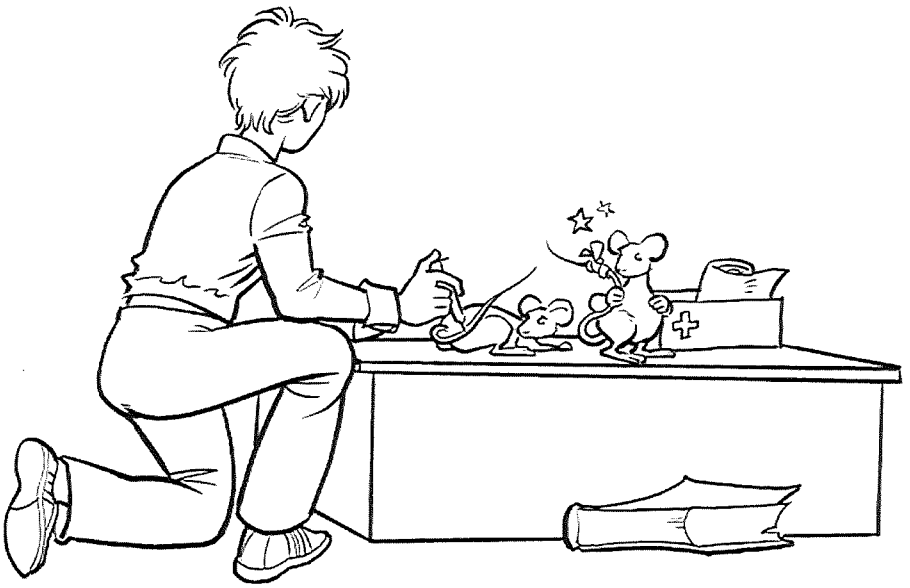


[1]these authors contributed equally to this work

The information of this chapter was submitted for publication.

Abstract

Apoptosis is an essential process ongoing throughout development and in tissue remodeling in the adult. It is also an essential process within the hematopoietic hierarchy, allowing tight regulation of subsets of hematopoietic cells. Overexpression of the anti-apoptotic Bcl-2 protein in transgenic mice has been a useful approach to study the role of apoptosis in several hematopoietic lineages. Previously, it has been shown that ubiquitous Bcl-2 overexpression in the hematopoietic system increases the viability and activity of hematopoietic cells under normal and/or stressful conditions in the adult. However, a role for apoptotic processes in the embryonic hematopoietic system has not yet been established. Therefore, we generated transgenic mice overexpressing *Bcl-2* under the control of the transcriptional regulatory elements of the hematopoietic stem cell (HSC) marker Sca-1. We show increased numbers and viability of Sca-1 positive cells in the aorta-gonad-mesenophros region (AGM), the first site of HSC generation. Moreover, we find increased HSC activity within the AGM and fetal liver. Together with data showing the normal expression of several pro-apoptotic and anti-apoptotic genes in the aorta, our results strongly suggest that apoptotic processes play a role in the development of HSCs.

Introduction

The hematopoietic system of adult mammals has its foundation in rare hematopoietic stem cells (HSC) harbored in the bone marrow. These cells are highly potent, contributing ultimately to the billions of mature hematopoietic cells in the blood and hematopoietic tissues throughout the lifetime of the individual [22,47]. In the mouse embryo, the first HSCs are autonomously generated in the aorta-gonad-mesonephros (AGM) at midgestation [27]. The onset of HSC activity occurs at embryonic day 10.5 (E10.5) and HSC activity increases until E14 in the liver [12,21,27,31,33]. Thereafter, the number of HSCs remains generally constant throughout life. The constant numbers of HSCs in the adult bone marrow are thought to be maintained through self-renewing cells divisions, in which one daughter cell retains HSC function, while the other differentiates along the hematopoietic hierarchy[10,41].

The signals and processes by which HSCs are generated, expanded and/or maintained in embryonic, fetal and adult stages are largely unknown. The fact that HSC numbers increase significantly in the midgestation mouse while remaining constant in the adult suggests that there are differences in the processes controlling HSC numbers at different times in ontogeny [21,27]. Although temporally-limited hematopoietic cell fate determination processes may be primarily responsible for generating more HSCs in the embryo, it is possible that the balance of programmed cell death and survival also plays a role in determining HSC numbers.

Programmed cell death or apoptosis, is a highly conserved process involved in tissue remodeling during development and adult tissue homeostasis (as reviewed 13,28). Apoptosis is characterized by a number of stereotypic changes in cell structure, including DNA fragmentation and changes in the plasma membrane which are mediated by members of the caspase protein family, ultimately leading to cell death (reviewed in 6,15). Evolutionary conservation of this process extends from *C.elegans* to mammals, as illustrated by the high degree of functional and structural homology between several apoptosis related proteins. Within mammalian embryos apoptosis is involved in the remodeling of the limbs, kidney and other structures [28,34]. Hence, the correct temporal and

spatial expression of these proteins is a prerequisite for normal tissue development and homeostasis [4,14,28,42].

The mammalian Bcl-2 protein family is divided into three subfamilies; the Bcl-2 subfamily which promotes cell survival, the pro-apoptotic Bax subfamily, and the pro-apoptotic BH3-only subfamily which includes Bim [1]. Based on several biochemical and cellular findings it has been proposed that pro- and anti-apoptotic proteins counteract each other's function via protein-protein interactions (reviewed in 3,49). Bcl-2 expression is found in immature hematopoietic and lymphoid cells, cells of the peripheral nervous system, epithelial cells and in the stem cells of the intestine [16,29]. Another pro-survival member of the Bcl-2 family, Bcl-x is also expressed within the hematopoietic system, nervous system and intestine [20,32]. Differences in the expression patterns of these two closely related proteins, suggest some non-redundant roles for Bcl-2 and Bcl-x *in vivo*. Since Bcl-2 is highly expressed in mature thymocytes [23] (as compared to Bcl-x expression in immature thymocytes [24]) it is thought to play a role in the thymocyte selection process. Indeed, overexpression of a *Bcl-2* transgene in T-cells increased cell survival and decreased negative selection[19,43,48].

Apart from the lymphoid system, the role of apoptosis in regulation of hematopoietic progenitor and stem cells is far less understood. A number of studies have investigated the role of apoptosis in HSCs using transgenic mouse models in which *Bcl-2* overexpression is directed using the hematopoietic specific *Vav* promoter [39] or by use of the more general/ubiquitous *H-2K* promoter [11]. In both cases, overexpression of *Bcl-2* within the HSC/hematopoietic progenitor compartment resulted in enhanced cell survival and radio-resistance of these cells [10,11,39]. However, the studies were limited to the analysis of adult and fetal hematopoietic cells, and did not study the effects of *Bcl-2* overexpression in AGM HSCs.

To more specifically analyze the role of Bcl-2 in HSCs during development, we describe the generation of transgenic mice overexpressing *Bcl-2* under the transcriptional control elements of the Sca-1 gene, *Ly-6E/A*. The Sca-1 cell surface protein is expressed on HSCs and is commonly used for the enrichment of HSCs [46,50]. We have previously demonstrated, using Sca-1 *LacZ* or *GFP* transgenic mice, that functional repopulating HSCs from adult bone marrow, fetal liver and midgestation dorsal aorta (the site of the first emerging HSCs) can be enriched significantly by flow cytometric sorting for β-galactosidase or GFP marker [7,25,26,30]. Thus, to study the role of apoptosis in the first HSCs within the embryonic dorsal aorta, we analyzed Sca-1 *Bcl-2* overexpressing transgenic mice. Here we show that Sca-1-directed *Bcl-2* overexpression results in increased numbers of Sca-1 positive cells in the AGM. The viability of these cells is increased and most importantly, AGM HSC activity is increased with Bcl-2 overexpression. Moreover, both anti-apoptotic and pro-apoptotic genes are normally expressed within the midgestation aorta as it is generating HSCs. These data strongly suggest that apoptotic processes play an important role in the regulation of HSCs beginning at the earliest stages of HSC development in the AGM.

Material and Methods

Generation of transgenic mice

An 865 bp murine *Bcl-2* cDNA fragment 36 was inserted in the *Ly6E.1* expression cassette [30,44,45]. The 14.9 kb NotI linearized fragment was microinjected into (CBAxC57BL/10)F1 oocytes Out of 17 founder mice born, 6 transmitted the transgene and two lines were used for further studies. Matings were set up between *Bcl-2* transgenic males and (CBAxC57BL/10)F1 females. The day of the vaginal plug was counted as day 0. Pregnant mice were killed by cervical dislocation and embryos were collected in PBS/ 10% FCS. Animals were housed according to institutional guidelines and animal procedures carried out in compliance with the Standards for Humane Care and Use of Laboratory Animals.

Genotyping, copy number determination and expression analysis.

For *Bcl-2* and *YMT* genotyping (Myo control), PCR conditions were: 100-200 ng DNA, 100 ng of each primer (Table 1), 1x buffer, 0.2mM dNTPs and 1U AmpliTaq (Perkin Elmer) with cycling: 5 min 92°C; 30x (40 sec 92°C, 40 sec 60°C, 1 min 72°C); 7 min 72°C. For copy number analysis a Southern blot with 10-20 µg HindIII digested genomic DNA was probed with a nick translated (Amersham Pharmacia Biotech) 32P-labelled 865 bp *Bcl-2* fragment. Analysis was performed by phosphorimaging (Molecular Dynamics) and ImageQuant software.

For Northern blot analysis 20 µg RNA isolated from several tissues with TRIZOL (Invitrogen/ Life Technologies) was blotted against Genescreen membrane (NEN life science products). The blot was probed with a 32P-labelled 865 bp *Bcl-2* cDNA fragment. *Ly6* and *GAPDH* probes are as described previously [30]. For RT-PCR analysis, embryos were (sub)dissected and tissues stored in RNAlater (Ambion) at 4°C. Embryos were genotyped and tissues from similar genotypes were pooled and homogenized in TRIZOL (Invitrogen/ Life Technologies). RNA was isolated according to manufacturer's instructions. 1-5 µg DNase treated RNA (RQ1 RNase free DNase Promega) was used for cDNA synthesis with Superscript II Reverse Transcriptase (Invitrogen/ Life Technologies). RT-PCR was performed in a 50µl volume with 100ng of each primer (Table 1), 0.2mM dNTPs, AmpliTag and buffer (PerkinElmer) together with a 40-100 ng RNA equivalent of cDNA. Cycling conditions: 5min 92°C, 28-30x (40sec 92°C, 40sec 58°C, 1 min 72°C), 7min 72°C. PCR products were run on 1.2% agarose/1xTBE gels with EtBr and scanned on a Typhoon scanner (Molecular Dynamics).

Table 1. Primer sequences

primers	primer sequences 5'-3'	based on sequence or reference	length PCR fragment
BC2PE	GTGCAGCTGACTGGACATCTCTGC	[30,35]	450
PE5	ACTCTGCCTGCAACCTTGTCTGAG		
YMT2-1	CTGGAGCTCTACAGTGATGA	[27]	342
YMT2-2	CAGTTACCAATCAACACATCAC		
Myo-1	TTACGTCCATCGTGGACAGC	[33]	245
Myo-2	TGGGCTGGGTGTTAGTCTTA		
mBcl-2 forw	GCACAGATGTCCAGTCAGCTG	NM_009741	268
mBcl-2 rev	GCCATATAGTTCCACAAAGGC		
mBcl-x forw	GGCGATGAGTTTGAACTGCG	U51278	915
mBcl-x rev	CCTCACTCAATGGCTCTTGG		
mBim forw	GAGAAGGTGGACAATTGCAG	AF032461	290, 380
mBim rev	GCCTTCTCCATACCAGACGG		
beta-actin forw	CCTGAACCCTAAGGCCAACCG	X03672	398
beta-actin rev	GCTCATAGCTCTTCTCCAGGG		

For Western blot analysis, protein extracts from several adult tissues were made in Laemmli buffer, separated on a 10 % SDS polyacrylamide gel and blotted to PVDF membrane (Millipore). Blots were blocked and incubated with an anti-Bcl-2 antibody (Alexis) in 2% milk/TBS-T followed by an anti-rabbit-HRP antibody (DAKO) and ECL detection.

Survival and cell viability assays.

Healthy transgenic and non-transgenic littermates of 7-13 (Ln 2) and 8-22 (Ln 479) weeks of age were randomly divided into 3 pools which received an equal split dose of 10.5, 9.5 or 8.5Gy gamma-irradiation. Mice received antibiotics and were checked daily for survival.

Whole bone marrow cells from *Bcl-2* transgenic and non-transgenic littermates were collected in PBS/10% FCS, suspended as single cells and counted (Coulter). Bone marrow cells were seeded at a density of $2x10^5$ cells/ ml in DMEM/10% FCS/Pen/Strep at 37°C, 5% CO_2. At 1, 2, 4, 8 and 11 days viable cell counts were determined by Trypan blue exclusion.

FACS analysis, immuno- and TUNEL staining.

AGM and liver tissues were dissected and explant cultures for 3-4 days were performed as described previously [21,27]. During the culture period, embryos were genotyped and tissues with similar genotypes pooled. Before staining, tissues were collagenase treated as described. Bone marrow cells were directly made into a single cell suspension. Cell staining was done in PBS/10% FCS with FITC or PE conjugated antibodies against Sca-1, c-kit, CD4, CD8, CD11b (Mac-1), Gr1, B220 and Annexin V (Pharmingen) and fluorescence measured on a FACScan or FACSVantage (Becton-Dickinson). Briefly, cells were stained with the antibody for 20 minutes on ice, washed and immediately measured. Dead cells were excluded by 7AAD or Hoechst 33258 (Molecular Probes) staining.

For immunofluorescence, single cell suspensions from AGM cells from *Ly6A-GFP* embryos made. Cytospin cells were fixed (2% paraformaldehyde/PBS) and stained with Bcl-2 antibody and α-rabbit-Texas Red or Cy3 secondary antibody.

Embryos were frozen in Tissue-Tek (Sakura) and cryosectioned (7-8 μM). TUNEL staining was performed with the *in situ* cell death detection kit/ POD (Roche) based on manufacturer's instructions. Staining was visualized with peroxidase substrate AEC and sections were dehydrated and embedded in Entallan (Merck)

In vivo repopulation assays for HSCs.

For all transplantations male donor cells were injected intravenously into (CBAxC57BL/10)F1 females irradiated with a 9.5Gy split dose of gamma-irradiation. Repopulation was assayed at 1 and 4 months post-transplantation by a Y-chromosome (*YMT*) specific PCR on peripheral blood DNA. Multilineage repopulation was analyzed on several recipients either by PCR on DNA from hematopoietic tissues or as described previously [8].

Donor male *Bcl-2* transgenic and non-transgenic littermates (10 weeks old) were sacrificed, bone marrow cells collected and injected into recipients at several cell doses in combination with 10^5 female competitor bone marrow cells. For repopulation assays on E11 and E12 AGM and liver cells, tissues were dissected and collagenase treated (0.125%) in PBS/ 10% FCS. 0.3 ee of AGM cells (~2 x 10^5 cells/ee for E11; ~3 x 10^5 cells/ee for E12) or 0.03 to 0.003ee of liver cells (~3 x 10^6 cells/ee for E12) were injected with $2x10^5$ female spleen cells into recipients. Only recipients injected with male embryonic cells were assayed for engraftment. For secondary transplantations, bone marrow was collected from primary recipients reconstituted at high levels and 10^5 cells were injected into secondary recipients. Secondary recipients receiving bone marrow from primary recipients injected with E12 AGM or liver were co-injected with 10^5 female bone marrow cells.

Results

Anti- and pro-apoptotic genes are normally expressed in the midgestation aorta.

To date, little is known concerning the expression of anti- and pro-apoptotic regulators in the midgestation AGM region and particularly HSCs. To determine if Bcl-2 is normally expressed in AGM HSCs, immunostaining was performed on cytospins of E12 *Ly-6A* (Sca-1) *GFP* transgenic AGM cells. Previous studies in such transgenic embryos have shown that all AGM HSCs are GFP^+ [7,26]. When we performed Bcl-2 specific antibody staining on such cytospins, we observed Bcl-2 expression in many AGM cells (Figure 1C), among which are GFP^+ aortic HSCs and mesonephric tubule cells (Figure 1B). Overlay of the two fluorescent signals shows that the GFP^+ cells co-express the Bcl-2 protein (Figure 1D). To more specifically determine if Bcl-2 is expressed in HSCs, cytospins of cells from subdissected E11 aortas were immunostained. Bcl-2 expression was found in many aortic cells (Figure 1G). A small number of GFP^+ cells indicative of HSCs were also found in the cytosypins (Figure 1F). Overlays of Bcl-2 and GFP signals demonstrate the co-expression of both molecules, strongly suggesting that E11 aorta HSCs express Bcl-2.

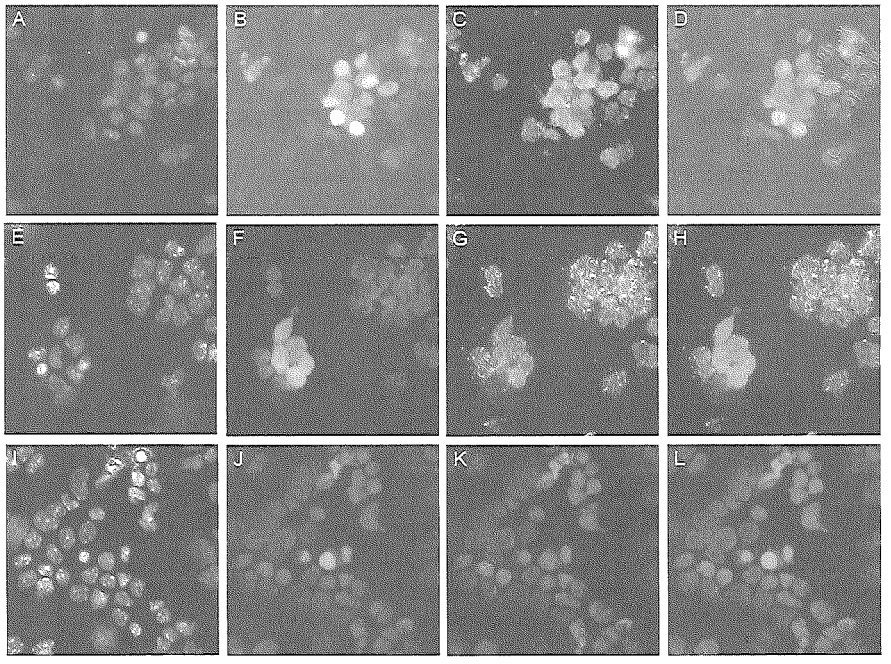


Figure 1. Sca-1+ AGM HSCs express Bcl-2.
Immunostaining was performed on Ly-6A (Sca-1) GFP transgenic E12 AGM and E11 aortic cells. AGM and aorta cells were isolated as a single cell suspension and deposited on microscope slides by a cytospin. Panels (A, E, I) DAPI staining; (B, F, J) Ly-6A GFP fluorescence; (C) anti-Bcl-2 Texas Red fluorescence, (G) anti-Bcl-2 Cy3 fluorescence, (K) incubated with no primary antibody. (D, H, L) overlay of GFP and Texas Red/Cy3 fluorescence. Panels A-D are a cytospin of E12 AGM cells stained with anti-Bcl-2 antibody conjugated with Texas Red. Panels E-H show a cytospin of E11 aorta cells stained with anti-Bcl-2 antibody conjugated with Cy3. Panels I-L show a cytospin of E11 AGM cells incubated with no primary antibody

Bcl-2 expression in AGM HSCs was further confirmed by RT-PCR analysis. AGM cells sorted on the basis of c-kit expression (a marker for HSCs but not mesonephric cells [40]) showed Bcl-2 expression in the c-kit$^+$ AGM fraction (not shown).

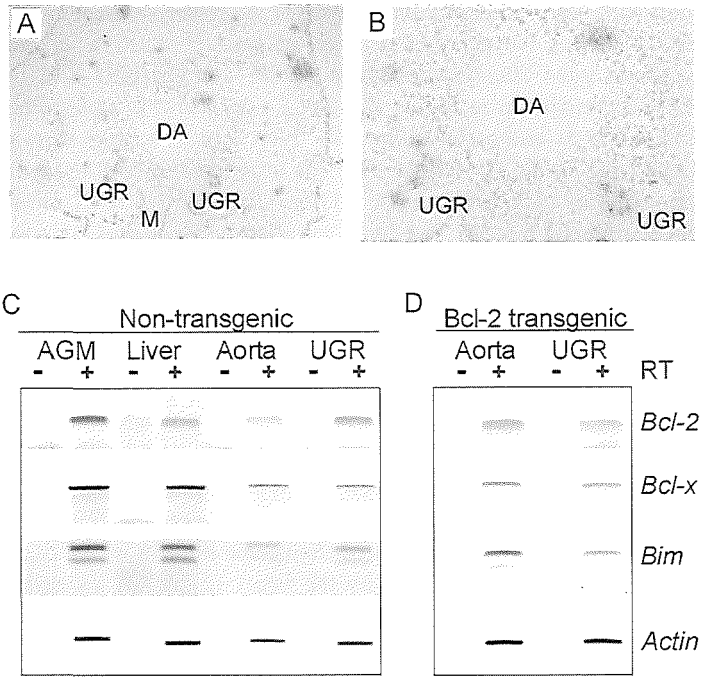


Figure 2. Apoptotic processes and molecules are present in the E11 AGM.
TUNEL straining was performed on transverse sections from the truncal region of an E11 embryo (Bcl-2 transgenic) to localize apoptotic cells in the AGM region. A representative section is shown in (A) 10x magnification and (B) 40x magnification. TUNEL positive (black/gray-colored) foci and diffuse scattered individual cells are observed. DA = lumen of dorsal aorta; UGR = urogenital ridges; M = mesentery. TUNEL staining was performed 3 times on 2 wild type and 2 transgenic embryos. No obvious difference in the TUNEL staining pattern was observed when sections from Bcl-2 transgenic embryos were compared with sections from non-transgenic embryos (not shown). (C) Expression of the anti-apoptotic genes Bcl-2 and Bcl-x and the pro-apoptotic Bim gene in the E11 AGM, aorta, UGRs and liver region of a non-transgenic embryo. RT-PCR analysis was performed on cDNAs made from the indicated midgestation tissues. Signal intensity from the actin PCR fragment was used as a normalization control. Note, that as expected, the Bim primers amplify two different transcript isoforms (splice variants). (D) RT-PCR analysis of a Bcl-2 transgenic embryo reveals altered levels of Bcl-2, Bcl-x and Bim gene expression in the E11 aorta and UGR. RT-PCR analysis was performed 3 times and confirmed on 3 independent sets of cDNAs.

To examine whether apoptosis normally occurs in the AGM, TUNEL analysis was performed on transverse sections through the truncal regions of midgestation embryos. Several TUNEL positive foci are found scattered throughout the representative E11 truncal section shown in Figure 2A. In the AGM region many highly positive foci are in the mesonephic tubules of the urogenital ridges (UGR) while only one strongly positive focus

was found a short distance from the dorsal side of the dorsal aorta (Figure 2B). Also, surrounding the dorsal aorta in the mesenchyme are diffuse individual weakly positive cells. Since we found a large number of AGM cells expressing Bcl-2 in the cytospins, the presence of TUNEL positive foci in AGM suggests that in these cells the balance is shifted towards the expression of pro-apoptotic regulators.

We next examined the expression levels of anti-apoptotic genes *Bcl-2* and *Bcl-x* and the pro-apoptotic gene *Bim* in the whole AGM, aorta, UGRs and liver by semi-quantitative RT-PCR. As shown in Figure 2C, *Bcl-2* expression is approximately two fold higher in the E11 AGM as compared to the E11 liver. Similar results were obtained with E12 tissues (not shown). In the E11 AGM, *Bcl-2* is expressed in both the aorta and UGRs, with the highest levels of expression in the UGRs. *Bcl-x* and *Bim* expression levels are comparable between the E11 AGM and liver and also between the E11 aorta and UGR subregions, although *Bim* expression appears to be slightly higher in the UGRs. Thus, both anti-and pro-apoptotic genes are expressed in the embryonic sites (aorta and liver) harboring the earliest HSCs. Taken together, these data indicate that apoptosis is taking place in discrete areas of the AGM, and although Bcl-2 is expressed in a majority of AGM cells, a regulated balance between cell survival and apoptosis is maintained within most of this midgestation tissue.

Sca-1 Bcl-2 transgenic mice overexpress Bcl-2 in hematopoietic cells.

To study whether apoptotic/cell survival processes are functional within the embryonic hematopoietic system, we generated transgenic mice in which overexpression of the anti-apoptosis gene *Bcl-2* was directed by the Sca-1 (*Ly-6E/A*) transcriptional control elements. A *Bcl-2* cDNA fragment[36] was inserted into the first untranslated exon of the 14 kb *Ly6E* genomic fragment[30,44] (Figure 3A) and two transgenic lines (Ln 2 and Ln 479) were analyzed in detail.

The copy number was determined by Southern blot analysis, comparing signal intensity of the endogenous 1.4 kb *Bcl-2* gene fragment to the 4.7 kb transgenic *Bcl-2* band (Figure 3B). The transgene copy numbers of Ln 2 and Ln 479 are 7 and 20, respectively. Northern blot analysis demonstrated overexpression of the *Bcl-2* transgene in kidney, liver (Figure 3C), thymus and spleen (data not shown). Non-transgenic littermates showed low, or undetectable levels of endogenous *Bcl-2* RNA. Western blotting revealed that Bcl-2 protein expression was also elevated in transgenic animals (Figure 3D). Only low or undetectable levels of endogenous Bcl-2 protein were found in wild type littermates. Thus, the Sca-1 transcriptional regulatory elements direct Bcl-2 expression at high levels in several hematopoietic and non-hematopoietic tissues in the transgenic animals and this expression pattern correlates with the *Ly-6E/A* expression pattern as previously reported [7,25,26,30].

Bcl-2 overexpression results in radio-protection and increased hematopoietic cell viability in the adult.

To test the validity of our transgenic model we examined the effects of Bcl-2 overexpression in the adult hematopoietic system. We performed survival experiments to assess whether Sca-1 directed *Bcl-2* overexpression could rescue cells from radiation induced apoptosis *in vivo*. Transgenic animals and normal littermates were challenged with different doses of total body gamma irradiation and the number of viable animals was

determined daily for up to 30 days. At doses of 10.5 (Figure 4A), 9.5 and 8.5 Gy (not shown) both Ln 479 and Ln 2 (not shown) transgenic animals showed higher survival rates at day 30 in comparison to their wild type littermates.

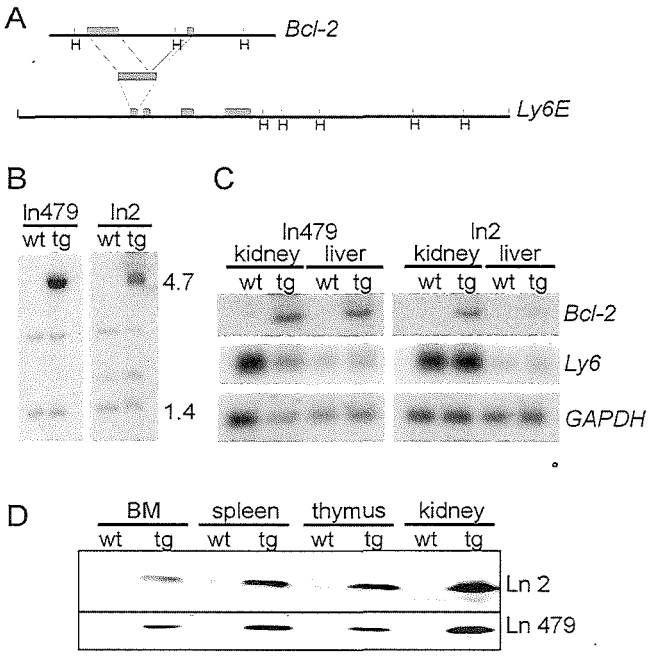
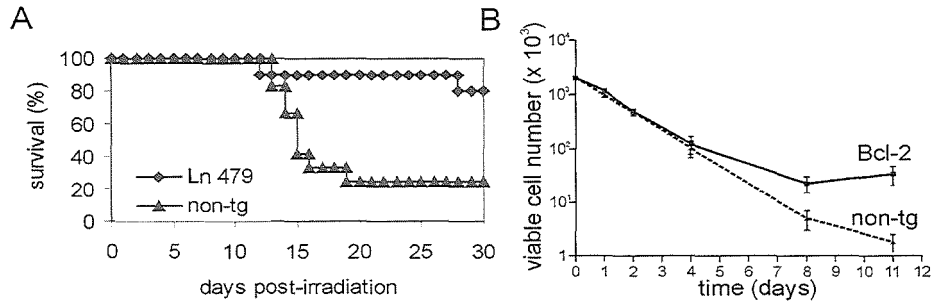


Figure 3. Sca-1 Bcl-2 transgenic mice overexpress Bcl-2 in hematopoietic tissues.
(A) Transgene construct. A 865 bp Bcl-2 cDNA fragment was inserted into exon one of a 14kb Sca-1 (Ly6E) expression cassette. H=HindIII. (B) Transgene copy number determination. A Southern blot containing HindIII digested DNA was probed with a Bcl-2 fragment to reveal the 1.4 kb endogenous gene and 4.7 kb transgene. Ln 479 contains 20 and Ln 2 contains 7 copies of the transgene. (C) Northern blot analysis for Bcl-2 transgene expression in several adult tissues. Bcl-2 is expressed in liver and kidney in Ln 479 and Ln 2 Bcl-2 transgenic (tg) animals. (D) Western blot analysis of Bcl-2 protein expression in Ln 2 and Ln 479 transgenic animals. Bcl-2 transgene is overexpressed in several tissues including kidney, thymus, spleen and bone marrow as compared to non-transgenic (wt) littermates. In general, no obvious abnormalities were found in the transgenic adults. Blood smears showed no morphologic differences or leukemic cells, and hematocrits did not differ between the transgenic and non-transgenic adults (data not shown).

To test whether this increased survival could be related to protection of cells of the hematopoietic system from apoptosis, we performed *in vitro* experiments using bone marrow (Figure 4B) cells from *Bcl-2* transgenic and wild type littermates. Over a period of 11 days in the absence of added hematopoietic growth factors, the survival of non-adherent cells was determined. The number of viable cells derived from *Bcl-2* transgenic bone marrow and spleen (not shown) was much greater than those derived from wild type littermates. Hence, the increased survival rate of the *Bcl-2* transgenic mice is due, at least in part, to increased viability of hematopoietic cells.



C

	Annexin - 7AAD -	Annexin+ 7AAD -	7AAD+
non-tg	58.3±4.7	10.5± 0.1	30.9±4.7
Bcl-2	91.0±2.5	3.7± 0.3	4.4±1.3

Figure 4. Effects of Bcl-2 overexpression on adult mice and cells.
(A) In vivo survival curves Bcl-2 overexpressing transgenic mice and non-transgenic littermates. Adult mice of 7-22 weeks of age were subjected to a split dose of gamma-irradiation of 10.5 Gy. Survival of both Ln 479 (shown) and Ln 2 (not shown) transgenic mice and littermates was checked daily over 30 days. Results are derived from 2 separate experiments for each transgenic line. The number of mice irradiated at 10.5 Gy was n=10 for Ln 479 tg and n=12 for wt littermates. For other irradiation doses (not shown), 10-15 mice were used per group. (B) Viable cell numbers of bone marrow cells of Ln 479 transgenic (Bcl-2) and a wild type littermate (wt). Bcl-2 overexpression results in higher viable cell numbers in vitro over a period of 11 days. Note that the scale on the Y-axis is logarithmic. These graphs show a representative experiment out of a total of 5 experiments (7 Bcl-2 tg and 7 wt littermates ranging 10 to 24 weeks of age). (C) Percentages of cells obtained after Annexin V and 7AAD FACS analysis of the bone marrow of 3 transgenic (n=2 Ln 2 and n=1 Ln 479, mice aged 9-23 weeks) and 3 non-transgenic littermates. Differences observed between the transgenic and non-transgenic in the Annexin-7AAD-, Annexin+7AAD+ and Annexin-7AAD+ subsets are statistically significant as determined by the students t-test.

We next examined bone marrow cells for stage specific involvement in the apoptotic process by flow cytometric analysis with Annexin V, an early marker of apoptosis, and 7AAD [53]. The results of three independent experiments in which non-transgenic cells were cultured under stressful hematopoietic growth factor-free conditions showed the expected pattern of cell death, with 31% of cells in the apoptotic and necrotic quadrants ($7AAD^+$). In contrast, *Bcl-2* transgenic cultures showed only 4% $7AAD^+$ cells, with a much higher percentage of cells in a viable state (91% $Annexin^-7AAD^-$). The percentage of cells in the early stages of apoptotis ($Annexin^+7AAD^-$) was almost 3-fold higher in the non-transgenic cultures as compared to the *Bcl-2* transgenic cultures. Thus, Bcl-2 decreases the number of BM cells entering into apoptosis in growth factor-free hematopoietic cultures.

To investigate whether lower apoptosis/higher cell maintenance was occurring in specific hematopoietic cell subsets we examined T and B lymphoid cells in the thymus and spleen (Table 2). Although the total body weight of *Bcl-2* transgenic adults was unchanged

from that of the wild type littermates, thymus cell numbers were slightly increased in the transgenic mice (but these changes did not reach statistical significance). We found increases in the percentages of $CD4^-CD8^-$, $CD4^+$and $CD8^+$ thymocytes and a concomitant large decrease in $CD4^+CD8^+$ thymocytes (Table 2). We also found that mature T cell numbers are increased in the spleen, with the absolute number of $CD4^+$ and $CD8^+$ splenocytes increased 2.5- to 4-fold. These changes are consistent with the expected expression pattern of the transgene and suggest that the most immature and mature subsets are maintained or positively selected [48] at the expense of the differentiating $CD4^+CD8^+$ thymocyte subset. Interestingly, the absolute number of spleen cells was increased a factor of 2.2 to 3.3, but this increase is not limited to T lymphocytes. In the spleen it was found previously that 10% of cells express Sca-1 [5] and that some of these cells are $B220^+$. Indeed the absolute number of $B220^+$ splenocytes was increased by 2 to 3-fold. Taken together, the increase in the absolute number of spleen cells (approximately 17-25 x 10^7) can be accounted for by the increase in the mature lymphocyte $CD4^+$ and $CD8^+$ and $B220^+$ subsets and may be attributed to increased cell survival by overexpression of Bcl-2. Thus, our transgenic animal model results in observable and expected Bcl-2 induced changes within the adult hematopoietic system.

Table 2. Lymphoid tissue and cell subset analysis of Bcl-2 transgenic adults

	Non-transgenic	Ln 479	Non-transgenic	Ln2
Body weight (g)	29.4 ± 5.7	29.4 ± 5.8	31.4 ± 4.0	32.0 ± 4.7
Absolute Cell Numbers (x 10^7)				
Thymus	7.7 ± 1.4	9.4 ± 1.1	10.8 ± 4.3	11.1 ± 3.6
Spleen	14.7 ± 1.6	31.9 ± 6.4	11.4 ± 1.1	37.3 ± 1.9
Subsets of Thymus cells (%)				
CD4-CD8-	3.7 ± 1.0	4.6 ± 1.7	2.0, 3.8	5.1 ± 1.7
CD4+CD8+	83.5 ± 3.6	75.6 ± 4.2	87.8, 83.3	70.9 ± 5.2
CD4+	9.9 ± 3.1	13.7 ± 2.0	11.9, 9.2	18.8 ± 2.4
CD8+	3.0 ± 0.6	6.1 ± 0.7	1.0, 0.9	5.2 ± 1.4
Subsets of Spleen cells (x 10^7)				
CD4+	3.1 ± 0.6	8.5 ± 1.1	2.8, 1.1	7.4 ± 1.9
CD8+	1.6 ± 0.5	4.0 ± 0.3	1.0, 0.5	2.8 ± 1.6
B220+	10.0 ± 0.7	21.4 ± 5.1	8.0, 2.9	18.6 ± 2.7

Tissues were isolated from mice ranging in age from 10-20 weeks. Both male and female mice are included in each of the groups. For the total thymus and spleen cell counts, sample numbers are as follows: Ln 479 mice (n=6) and non-transgenic littermates (n=6); Ln 2 mice (n=11) and non-transgenic littermates (n=7). For the spleen and thymus subset cell determinations n=3 except for non-transgenic Ln 2 control group.

Bcl-2 overexpression results in increased numbers and viability of Sca-1 positive cells in the AGM.

The first HSCs emerge in the AGM region beginning at E10.5 and are increased in number by more than 15–fold when this tissue is cultured as an explant for 3 days. To determine whether apoptotic/cell survival processes are functional in these AGM HSCs and are affected by Bcl-2 overexpression, we examined the numbers of Sca-1$^+$ cells in E11 AGM control and transgenic explants by FACS analysis. As shown in Figure 5A, the percentage of Sca-1$^+$ cells is increased in the *Bcl-2* transgenic AGMs as compared to non-transgenic AGMs. A 1.4- to 2.2-fold increase was consistently observed in transgenic AGM explants (Figure 5C) and moreover, even greater increases in the percentages of Sca-1$^+$ cells in E11 transgenic liver explants were observed (1.7 to 3.9 fold; Figure 5C). To examine whether this increase in Sca-1$^+$ cell number is related to changes in apoptotic processes, we tested for the expression of Annexin V within the Sca-1$^+$ fraction of AGM

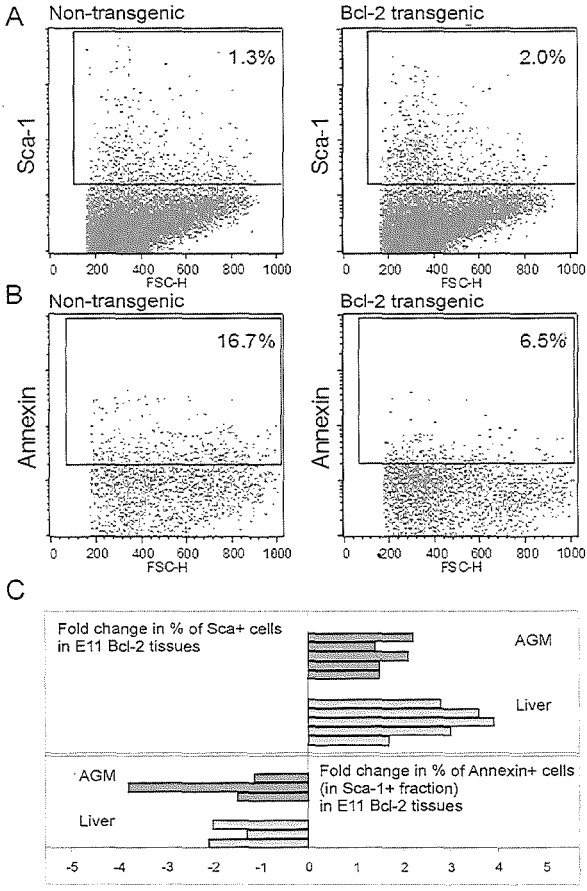


Figure 5. Increased numbers and viability of Sca-1$^+$ cells in the AGM and liver.
E11 AGM tissue was dissected and culture in an explant culture system for 3-4 days during which the embryos were genotyped. AGM tissue from embryos with a similar genotype were pooled and single cell suspensions made and stained for Sca-1 and Annexin V. (A) representative FACS plot for Sca-1 in non-transgenic and Bcl-2 overexpressing AGM tissue shows that Bcl-2 overexpression results in increased numbers of Sca-1$^+$ cells. Number of events in non-transgenic plot = 1.5 x10^4 and in transgenic plots= 1.3x10^4. (B) Within the Sca-1 positive fraction, the percentage of Annexin V$^+$ cells was determined as indication for the amount of apoptotic cells. Bcl-2 overexpressing tissues contain less AnnexinV$^+$ cells within the Sca-1$^+$ fraction after the culture period. Number of events in non-transgenic plot = 2.1 x10^3 and in transgenic plots= 1.7x10^3. (C) Combined results of total 5 experiments for Sca-1 staining and 3 experiments for Annexin V staining of the Sca-1$^+$ fraction of E11 AGM cells.

and liver cells. As shown in Figure 5B, the percentage of Annexin V^+ cells is decreased in the *Bcl-2* transgenic AGM explant. This decrease ranged from 1.4- to 3.8 fold in *Bcl-2* AGM explants and 1.3- to 2.0-fold in liver explants (Figure 5C). Thus, Bcl-2 overexpression leads to a quantitative increase in HSC numbers by decreasing apoptosis of Sca-1^+ cells in midgestation hematopoietic tissues.

As the overexpression of Bcl-2 increases Sca-1^+ cell survival in the embryo, we sought to determine whether the expression levels of the anti-apoptotic Bcl-x and pro-apoptotic Bim genes were also affected. Semi-quantitative RT-PCR analysis of *Bcl-2*, *Bcl-x* and *Bim* expression in midgestation embryonic transgenic tissues (Figure 2D) showed that not only was *Bcl-2* gene expression increased (2-10 fold), but also *Bim* expression increased (approximately 1.5-4 fold) when transgenic aorta was compared to non-transgenic aorta. *Bcl-x* expression remained as in the non-transgenic. Interestingly, RT-PCR analysis of non-transgenic AGMs prior to and after explant culture, showed increased Bcl-2 and Bcl-x expression after 3 days of culture. The level of Bcl-2 and Bcl-x remained the similar between cultured and non-cultured liver (C.O. and E.D., manuscript in preparation). Taken together, these data strongly suggest that anti-apoptotic pathways are in place, functioning and regulated at the level of gene transcription in a balanced manner.

Hematopoietic stem cell activity is increased in the AGM of Bcl-2 overexpressing embryos.

To test the validity of our animal model for Sca-1 directed *Bcl-2* overexpression in HSCs, we first compared bone marrow from transgenic mice with non-transgenic bone marrow for *in vivo* hematopoietic repopulation. More HSC activity was found in *Bcl-2* transgenic bone marrow (Figure 6A) at a limiting dose of 10^4 cells than in wild type littermate controls. HSC frequencies were calculated (Table 3) and found to be increased by 2.6-fold ($p<0.03$) in the Bcl-2 overexpressing mice. Moreover, by flow cytometry on *Bcl-2* transgenic bone marrow ($n=3$), we found a 1.6- to 3.7-fold (2.6 ± 1.1) increase in the number of c-kit^+ cells as compared to non-transgenic bone marrow (not shown). Thus, Bcl-2 overexpression increases the number of bone marrow HSCs indicating the validity of our transgenic model.

To directly test whether Bcl-2 overexpression promotes the viability/survival of HSCs in the midgestation hematopoietic tissues, we performed limiting dilution *in vivo* transplantation experiments. AGMs and livers were obtained from transgenic and wild type male embryos at E11 and E12 and injected into irradiated female recipients. Recipients were analyzed for donor cell Y chromosome contribution by peripheral blood DNA PCR. At 4 months post-injection the HSC activity of E11 *Bcl-2* transgenic AGM cells (Figure 6B) was higher (10 repopulated of 25 recipients) than that from non-transgenic littermate controls (only 2 of 19 recipients repopulated). Engraftment was high level (up to 100% donor-derived), long term and multilineage (not shown). By E12, HSC activity in transgenic AGMs was similar to that of the E12 non-transgenic AGM, but this may be due to saturating numbers of HSCs in the injected dose. *Bcl-2* transgenic E12 liver also showed some increases in HSC activity when compared to non-transgenic liver at limiting injection doses (Figure 6C). Frequency analysis (Table 3) revealed that HSC numbers were significantly increased in the E11 AGM by a factor of 4.5 ($p<0.02$).

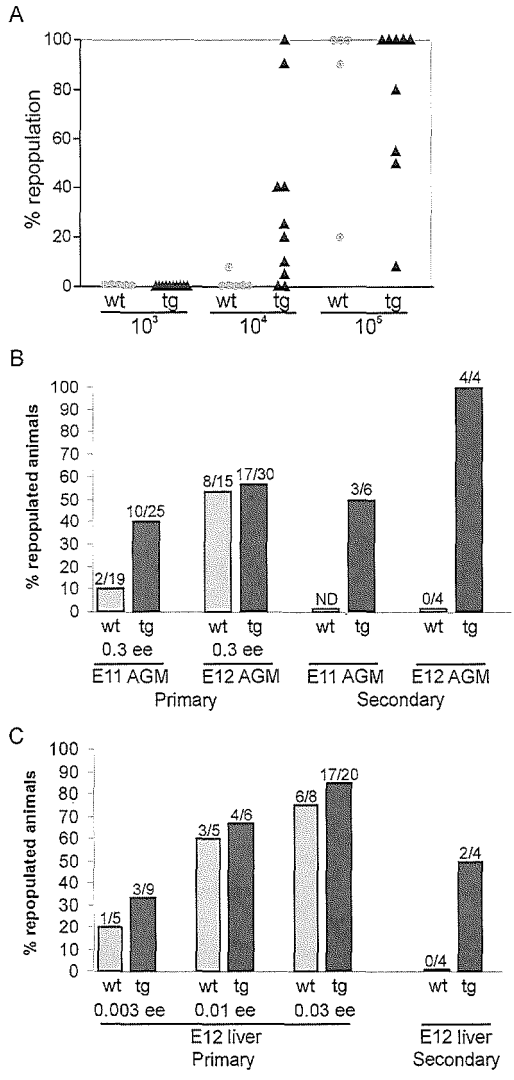


Figure 6. Increased HSC activity in the bone marrow, AGM and liver of Bcl-2 overexpressing transgenic animals.

(A) HSC activity in the bone marrow of Bcl-2 overexpressing mice. Whole bone marrow from a male Ln 479 transgenic (tg) and a non-trangenic (wt) littermate was injected at different limiting doses (10^3, 10^4, 10^5 cells) together with 10^5 female bone marrow cells into irradiated female recipients. After 4 months post-transplantation the peripheral blood of the recipient was analyzed in a semi-quantitative manner for male donor hematopoietic cells. Black triangles represent individual recipients engrafted with tg bone marrow and grey spheres individual recipients engrafted with wt bone marrow. Results of competitive repopulation experiments for Ln 479 transgenic and non-transgenic littermates are shown. A 10% engraftment level in the peripheral blood was used as the criterion for positive HSC repopulation. Ln2 trangenic bone marrow gave similar results (not shown). Transplantations were performed with (B) E11 AGM, E12 AGM Ln 479 transgenic (black bars) and (C) E12 liver cells (black bars) as well as non-transgenic control cells (light bars). The combined results of two independent transplantation experiments show the percentage repopulated recipients (y-axis) and the number of mice positive/the number of total mice injected for each group. ee=embryo equivalents injected. All recipients considered positive at >4 months post transplantation showed donor-derived cell engraftment levels greater than 10% by semi-quantitative PCR. In general, a higher percentage of repopulated recipients is seen with Bcl-2 transgenic (tg) midgestation AGM and liver cells than with wild type (wt) littermate cells. Results of secondary transplantations are shown in the right panels of (B and C). Bone marrow cells from primary recipients of E11 AGM, E12 AGM and E12 liver were transplanted in limiting dilutions into irradiated adult secondary recipients. Percentage repopulated recipients and the number of mice positive/the number of total mice injected is shown for each group. The bone marrow cells of the primary recipients of E11 AGM and E12 AGM and E12 liver cells that were used for secondary transplantation were 75-100% repopulated by donor-derived cells. Results were obtained at >4 months post transplantation by PCR analysis of peripheral blood DNA. ND=not done. Again, a higher percentage of repopulated recipients is seen with Bcl-2 transgenic (tg) as compared with wild type (wt) littermate cells.

To further check the potency of these HSCs, limiting doses of bone marrow cells from the primary *Bcl-2* transgenic and non-transgenic AGM- and liver-engrafted recipients were injected into secondary recipients. *Bcl-2* transgenic secondary transplanted AGM cells showed potent HSC activity (Figure 6B, right panel) while non-transgenic cells provided no engraftment (combined data of transgenic secondary transplants, 9 repopulated of 14 transplanted as compared to non-transgenic transplants, 0 of 8). Similarly, *Bcl-2* transgenic secondary transplanted liver cells showed HSC activity while non-transgenic liver did not engraft (Figure 6C, right panel). Thus, these transplantation data demonstrate that Bcl-2 overexpressing embryos possess higher HSC activity in the AGM region and liver than non-transgenic embryos and suggest that Bcl-2 survival processes are in place and fully functional in these HSCs at the earliest stages of development.

Table 3. Frequency analysis of HSCs in embryonic and adult hematopoietic tissues

Tissue	Non-transgenic		Bcl-2 transgenic	
Transplanted	HSC frequency	HSC/ee	HSC frequency	HSC/ee
E11 AGM	1/ 2.7 x 10^5	0.37	1/ 0.6 x 10^5	1.70 *
E12 AGM	1/ 0.4 x 10^5	2.54	1/ 0.4 x 10^5	2.78
E12 liver	1/ 0.017 x 10^5	58.8	1/ 0.013 x 10^5	76.9
Bone marrow	1/ 6.28 x 10^4	-	1/ 2.31 x 10^4	-

*HSC frequencies were determined from the primary transplantation data in figure 6 and were calculated using the L-Calc software. *statistical significance ($p<0.02$). ee=embryo equivalent*

Discussion

It has been widely accepted that a tight quantitative control of HSCs is essential to maintain the appropriate and stable representation of progenitors and differentiated cells in the adult hematopoietic hierarchy. Because the blood system is highly dynamic, must respond rapidly to trauma and must also constantly replenish itself, cell survival and apoptotic processes play an important role in regulation of this tissue system. During development of the hematopoietic system in the embryo these processes also appear to play a role. We have shown here that Bcl-2 overexpression increases the numbers of Sca-1^+ cells in the AGM, decreases apoptosis of these cells and more importantly, increases the numbers of functional HSCs beginning at midgestation in the AGM.

HSC activity was significantly increased in the E11 AGMs from Sca-1 *Bcl-2* transgenic embryos (4.5-fold increase, $p<0.02$). Such an increase was less apparent in transgenic E12 AGM and liver. However, when secondary transplantations were performed to confirm the self-renewal ability of AGM and liver HSCs, increased HSC activity was observed in the *Bcl-2* transgenic primary bone marrow as compared to that from the non-transgenic recipients, suggesting that saturating numbers of HSCs were present in the primary Bcl-2 recipient. Thus, Bcl-2 overexpression affects the repopulating activity of the first AGM and liver HSCs. Whether Bcl-2 overexpression has a direct effect only on HSC number or has several related effects, such as enhancing engraftment through survival and/or influencing the cell cycle status of HSCs (as for adult bone marrow HSCs), is yet to be determined. Importantly, whatever the effect of Bcl-2 overexpression on HSCs, these cells are normal, contributing to long-term, multilineage repopulation and most-likely

enhanced self-renewal. The fact that increased HSC numbers are found in the AGM, liver and the adult bone marrow suggests that intriguing possibility that HSCs apoptose more often and much earlier than previously appreciated.

It can be implied from the observed HSC increases in Sca-1 *Bcl-2* transgenic mice that components of Bcl-2 pathway leading to cell survival are intact and functionally relevant in these cells. Until our investigation, it was not known whether *Bcl-2* is normally expressed in this region. We have shown that Bcl-2 protein is expressed in the midgestation AGM and furthermore, that some apoptosis is ongoing in this region. *Bcl-2* transcripts are found both in the aorta and the UGRs, and more specifically in aorta HSCs. While TUNEL positive foci are found in the mesonephric tubules (indicating apoptosis), it appears that apoptosis occurring in the region surrounding the dorsal aorta is limited to single cells. These data suggest that there is a tight regulation of apoptosis in this region and in addition to Bcl-2, we postulate that other apoptotic regulators play a role in the balance between cell survival and apoptosis in the AGM.

We have found expression of the Bcl-x anti-apoptotic regulator and the Bim pro-apoptotic regulator in the midgestation aorta and UGRs. Since the results of others in adult hematopoietic cell subsets and tissues [20] show only slight overlap in Bcl-2 and Bcl-x *in vivo* expression patterns, these molecules may be expressed in different AGM cell subsets. Indeed, gene targeted mutant mice show different phenotypes [32,34,52]: *Bcl-2* deficient mice complete embryonic development and show increased apoptosis in lymphocyte populations, and *Bcl-x* deficient mice die around E13 with extensive apoptosis of hematopoietic cells in the fetal liver. Interesting, recent data show that concomitant loss of the pro-apoptotic *Bim* gene and the anti-apoptotic *Bcl-2* gene allows normal physiological functions and thus, Bim counterbalances Bcl-2 activity [2]. Moreover, *Bim* expression has been found in several hematopoietic cell lines [17,37] and *Bim* upregulation correlates with increased apoptosis induced in E17 fetal liver cells upon hematopoietic growth factor withdrawal [9]. Therefore, there are important roles for Bcl-2, Bcl-x and Bim in regulating apoptosis in hematopoietic cells and the intracellular ratios between these and other pro- and anti-apoptotic proteins are expected to play a critical role in setting the balance between cellular life or death. Semiquantitative transcriptional analysis of these three genes in the normal and Bcl-2 overexpressing AGM, aorta, UGRs and liver suggests that the balance of pro- and anti-apoptotic molecules is also important during midgestation hematopoiesis. When *Bcl-2* is overexpressed in aorta in transgenic embryos, a concomitant increase in *Bim* but not *Bcl-x* is observed. How this balance is achieved at the transcriptional level is unknown. Further studies should determine whether these three molecules (or other combinations) are expressed in the same population/subset of AGM cells and the precise balance between these pro- and anti-apoptotic molecules that leads to increased HSC activity in the mouse embryo.

Finally, in examining the validity of our *Bcl-2* overexpression transgenic mouse model in the adult hematopoietic system, we found that Bcl-2 overexpression occurs in the tissues and hematopoietic cells that are normally expected to express Sca-1 [7,25,26,30]. Sca-1 directed *Bcl-2* overexpression affects adult hematopoietic cells, both under normal and stressful conditions, most likely by preventing them from entering the apoptotic pathway. As expected from the previous results of others [11,48,48], Sca-1 directed *Bcl-2* overexpression results in radio-protection and in increases of bone marrow HSC activity (as determined by *in vivo* transplantation). Frequency analysis revealed that bone marrow HSC numbers were

significantly increased in *Bcl-2* transgenic adults by a factor of 2.7. This increased bone marrow HSC activity may be related to a lower susceptibility of the transgenic HSCs to enter into apoptosis during the engraftment of the recipient. Several studies have reported that Bcl-2 influences the cell cycle by blocking the transition from G_1 to S-G_2-M [38,51]. Since there is a correlation between the cell cycle status of HSCs and their ability to home to the recipient bone marrow (18 and references therein), it is possible that altered cell cycle profiles could result in the facilitated engraftment we observed for Sca-1 *Bcl-2* overexpressing HSCs. Notwithstanding, the Bcl-2 related increase in HSC activity is also due to an increase in the absolute number of HSCs, since we have found more c-kit$^+$ cells in *Bcl-2* transgenic bone marrow. Interestingly, both the bone marrow and AGM contained approximately 2-fold more HSCs. Given the notion that Bcl-2 overexpressing HSCs enter into apoptotic stages at a much lower frequency and remain viable for longer periods of time, it may now be possible to isolate cell lines of this difficult to culture Sca-1$^+$ hematopoietic subset from the AGM and adult bone marrow. Such cell lines would greatly facilitate molecular analyses of developmentally specific HSC genetic programs.

Taken together, our studies show that anti- and pro-apoptotic processes are intact, active and functioning in the earliest expanding HSCs in the AGM region of the mouse embryo and that apoptosis/cell survival plays an important role in these first HSCs.

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Chapter 6

General Discussion & future prospects

During mouse development hematopoietic cells can be found in several tissues in a distinct spatial and temporal manner. The first hematopoietic cells can be found in the extra-embryonic yolk sac from E7.5 onwards. These primitive, nucleated erythrocytes are later in midgestation development replaced by definitive, enucleated erythrocytes[34]. The first definitive adult-repopulating hematopoietic stem cells (HSCs) can be detected and are autonomously generated in an E10.5 intra-embryonic region, referred to as the aorta-gonad-mesonephros (AGM) region. Slightly later, from E11 onwards, these HSCs also become detectable in the circulating blood, the liver and the yolk sac[25,30,32]. Further mapping revealed that the first detectable HSC activity in the AGM is confined to the aorta region at E11[10,11]. Moreover, HSCs can expand within the AGM region upon organ culture, suggesting that the AGM harbors a unique micro-environment with the ability to support hematopoietic stem cell emergence and/or self-renewal (proliferation)[15,25]. Although a large number of genes have been implicated in the regulation of hematopoietic stem cells during development, a complete and comprehensive understanding of these regulatory processes is still lacking.

Differentially expressed genes in the AGM region between E10 and E11 could represent regulators of HSC activity.

To gain further insight in the emergence and regulation of the first detectable adult-repopulating HSCs in the AGM region we set out to identify genes that were differentially expressed between E10 and E11 of mouse gestation, as described in chapter 2. These time points were chosen because at E10 there are no HSCs in the AGM region while at E11, AGM HSCs are abundant. The AGM region is a complex tissue, consisting of the kidney anlagen and gonads as well as the dorsal aorta and surrounding mesenchyme. To increase the probability of detecting genes specifically involved in HSC regulation, the AGM region was subdissected to yield the aorta/mesenchyme region, the subregion where the first HSCs are found. A differential gene expression screening was performed using the aorta/mesenchyme tissues derived from E10 and E11 embryos. Additionally, two other screens, one with complete AGMs and one with complete E10 AGM as compared to E11 aorta/mesenchyme subregions, were performed to find differentially expressed genes.

Currently, differential screenings are routinely performed with micro-array screening methods, in which a large number of genes can be screened simultaneously for differential expression between two populations of cells. However, at the onset of the studies for this thesis, micro-array screening methods were still very expensive and more importantly, the technique was still in its infancy. Therefore, a differential screening method developed in 1992, Differential Display RT-PCR[28] was used to search for differentially expressed genes. Despite the known drawbacks of this technique, such as high number of false positives [27,29], and the fact that we were searching for differentially expressed genes in a complex tissue, we were able to identify and verify a number of truely differentially expressed genes. Among the identified and verified differentially expressed genes we detected two novel mouse genes, *Tab2* and *Tm9sf2*. In addition, we found that an essential component of the Wnt signaling pathway, *β-catenin*, was differentially expressed. Besides these genes, that were further studied, other interesting genes were identified in the screen. Expression of a zinc finger protein encoding gene, named *Sag*, proposed to function as an apoptosis regulator[14,42], the *Bub1b* gene implicated as a mitotic spindle checkpoint regulator[46], and *Myc* and *Rb binding protein* encoding genes were found in the midgestation

AGM region. However, as it was not possible to further investigate all the interesting genes identified, my primary research focus was on the novel gene, *Tab2*, its role in the IL-1 signaling pathway, and the role of IL-1 signaling in HSC development. I also examined expression of other Wnt signaling members in midgestation hematopoietic tissues.

Wnt signaling components in the AGM

Expression of Wnt signaling components was investigated by RT-PCR analysis on E10-E12 AGM and liver material. We observed that transcription of several components of the Wnt signaling pathway, including *Disheveled (1, 2 and 3)*, *Wnt5a* and *Wnt3,* is initiated and upregulated between E10 and E11 in the AGM region. Also, other essential components, such as, *β-catenin*, *Lef, Tcf1* and *Tcf3,* are expressed in the E11 AGM. Thus, several important mediators of the Wnt signaling pathway are present at the molecular level and their presence suggests some function in hematopoietic development. Although the functionality of Wnt signaling in the midgestation AGM and liver was not investigated, several studies by others have implicated the importance of Wnt signaling and regulation of hematopoiesis and HSCs. For example, Austin et al. [4] reported that Wnt5a is expressed in the yolk sac and the fetal liver and *in vitro* studies showed that Wnt5a could expand fetal liver hematopoietic progenitors. Moreover, it was recently reported that Wnt3 can act as a HSC growth factor and was proposed to be an important *in vivo* regulator of HSC self-renewal [4,35,48]. Together these studies implicate an important role for Wnt proteins in HSC regulator and based on their expression in the E11 AGM region, these proteins might play a role from the earliest stages of HSC development onwards. However, to study the functionality of Wnt proteins in the AGM raises several technical difficulties. The most obvious manner to examine the impact of Wnts on AGM hematopoiesis would be to perform organ cultures or to culture purified AGM HSCs in the presence of Wnt proteins and subsequently determine the effect by *in vivo* transplantation and *in vitro* colony forming assays. This method encounters the problem that correctly folded Wnt proteins are not easily purified. Moreover, Wnt proteins need to interact with the extracellular matrix in order to be presented correctly to the cells [9,49]. A more elegant, but also more time-consuming way to investigate the role of *Wnt* genes on AGM HSCs would be to investigate the characteristics of AGM HSCs in mouse models that either overexpress or are deficient for *Wnts* and/or Wnt signaling components. Although the role of Wnt signaling in the AGM was not further addressed here, the fact that these genes are expressed at the time that HSC activity becomes detectable might serve as a starting point for future investigations concerning the role of Wnt signaling in AGM HSC regulation.

TAB2 expression pattern in the mouse

Besides the identification of Wnt signaling components that were differentially expressed in the AGM region between E10 and E11, we also identified two novel mouse genes homologous to known human genes. One of these mouse genes encoded for a transmembrane protein with unknown function and the other gene encoded for *Tab2*. Human TAB2 had been previously implicated as a mediator of interleukin-1 (IL-1) signaling[44]. As IL-1 has been reported to regulate HSCs in the adult mouse, it was interesting to further investigate the role and function of TAB2 within the hematopoietic system. Therefore, we set out to perform several studies regarding the expression pattern of

TAB2 and related genes as well as the possibility of IL-1 as a regulator of HSCs in the AGM and fetal liver.

TAB2 was originally identified as a TAK1 binding protein and TAK1 itself has been identified as a kinase in the TGFβ signaling pathway[44,51]. Besides TAB2, two other TAK1 binding proteins have been identified, TAB1 and TAB3[33,40]. Soon after our identification of *Tab2,* a full length mouse *Tab2* IMAGE cDNA clone became available. Alignment of the predicted protein sequence of the *Tab2* IMAGE cDNA clone with the hTAB2 protein sequence revealed 95% similarity between these two homologous proteins. High similarity was also observed upon alignment of TAK1 and TAB1 protein sequences between several species, including mouse, human, *Xenopus*, *C.elegans* and *Drosophila* suggesting a conserved function for these proteins. Indeed it has been reported that TAK1 (and TAB1) play an important role in the conserved IL-1/Toll signaling pathway in the innate immune system and also in the conserved BMP and Wnt signaling pathways[22,39,41,47]. However, little is known regarding the function and expression pattern of TAB2. As the protein structure of TAB2 and TAB3 is highly similar, these proteins could fulfill a redundant or overlapping function. Therefore, we examined their RNA expression pattern in several adult mouse tissues and cell lines, as described in chapter 3. In the tissues tested, we found a similar expression pattern for *Tab2* and *Tab3*. However, in the clonal cell lines we tested, we did not observe a clear overlap in expression patterns, suggesting that these genes are not expressed in similar cell types and might also be regulated differently. This further implies that the genes *Tab2* and *Tab3* are expressed *in vivo* in different cell types and might have distinct functions. However, as no protein expression analysis could be performed for TAB3, due to lack of a TAB3 specific antibody, the *in vivo* expression pattern remains to be elucidated. For TAB2 we did perform protein expression analysis in the adult and the midgestation embryo. By Western blotting, we found ubiquitous expression, with lowest expression levels in the hematopoietic tissues. This expression pattern of TAB2 is similar to that reported for IL-1R1 expression which was shown to be expressed to low levels in hematopoietic tissues and to high levels in other non-hematopoietic tissues [13]. As the Western blot analysis does not provide insight into spatial expression of TAB2 in tissues, we also performed immunohistochemistry.

Immunostained adult tissue sections revealed TAB2 expression patterns in cell subsets in the thymus, spleen and bone marrow. TAB2 expression was also detected in cell subsets in the brain and lung. Interestingly, the TAB2 expression pattern observed in adult mouse tissues is strikingly similar to the pattern observed in a NFκB reporter mouse, as published by Schmidt-Ullrich et al. [37]. In this reporter mouse, the *Nfκb1* promoter drives *lacZ* expression. In addition, an artificial promoter construct was used containing three consensus *Nfκb* sites was used to drive *lacZ* expression in another reporter mice. Since those promoters contain NFκB binding sites, it is generally accepted that *Nfκb* activity can be monitored by examining the lacZ expression pattern in adult and embryonic tissues. However, some small differences in expression pattern may be induced by or repressed by other transcription factors or by the different NFκB dimmers. Notwithstanding, it is very interesting that the expression pattern of lacZ in these *Nfκb* reporter mice is similar in the adult brain, thymus and spleen to our TAB2 staining pattern. Moreover, the lacZ staining observed in the embryonic brain, spinal cord and some of the blood vessels, is also similar to the observed TAB2 expression pattern. The only important difference is the absence of lacZ expression in the fetal liver, in all but one transgenic mouse line. Whether the lacZ

expression is below detection or whether NFκB activity is not present in this tissue is unclear. However, an important role for NFκB signaling in liver development has been suggested based on the reports of mice deficient for the NFκB member *RelA* and *IκB kinase 2* (*Ikk2*). Such deficiencies are embryonic lethals due to fetal liver degeneration by apoptosis [7,26]. However, *Nfκb1* (p50) deficient mice are not embryonic lethal [38] and this is explained by the fact that p50 itself lacks transactivating properties and instead modulates the activity of other NFκB family members[7,12,26]. Nevertheless, the similarity of expression pattern of this NFκB family member and TAB2 and suggests that TAB2 and NFκB signaling might be intimately linked.

Interleukin-1 and the regulation of HSCs

Previous studies implicating IL-1 as a regulator of proliferation, differentiation, migration and as radio-protector of HSCs in the adult bone marrow, together with our interest in TAB2, prompted us to investigate the expression of the IL-1 receptor and its signaling components in the midgestation AGM region and liver, and more specifically the effect of IL-1 on E11 AGM and liver HSCs. As described in chapter 4, we observed expression of IL-1 signaling components and could even demonstrate an increase in HSC activity upon addition of IL-1 in organ cultures of AGM or liver tissue. The finding that the IL-1RI is expressed in/near the endothelium of the dorsal aorta in the E11/ E12 AGM region and that the IL-1RI is expressed on c-kit expressing cells in the AGM and liver, suggest that IL-1 is an *in vivo* regulator of midgestation HSCs. To confirm that the IL-1RI expressing cells are truly HSCs, FACS sorting experiments and in combination with long-term repopulation assays need to be performed. Nevertheless, whether acting directly or indirectly on HSCs, it is clear that IL-1 is a potent positive regulator of HSC activity during organ cultures.

Currently, it is unclear how IL-1 increases AGM HSC activity. It may act to decrease apoptosis, increase proliferation or to alter migration/ adhesion properties of HSCs. Further studies investigating these possibilities are required to determine the nature of the IL-1 induced changes in the AGM. For example, to examine whether IL-1 plays a role in proliferation or apoptosis during the organ cultures, FACS analysis performed on AGM tissue cultured in the presence or absence of IL-1 would determine whether HSC (i.e. c-kit$^+$/ CD34$^+$/ Sca$^+$ cells) numbers increase or decrease. In addition, BrdU incorporation studies could be performed to monitor proliferation of HSCs. Moreover, FACS analysis for the apoptosis marker Annexin and TUNEL stainings can be used to monitor apoptosis in AGM and liver cells. Also by gene expression analysis of cultured tissues for known apoptosis regulators, such as *Bcl-2* family members, could reveal IL-1 induced changes.

The effects of IL-1 on migration and adhesion are less easily monitored, although there is an indication that IL-1 might indeed be regulating these processes. By expression analysis on cultured AGM and liver tissue in the presence of IL-1 we found that *Matrix metalloprotease-9* (*Mmp-9*) is strongly upregulated. Recently, it was reported that MMP-9 plays an important role in regulating the amount of soluble SCF in the bone marrow and thereby influencing the proliferation and migration of HSCs[19]. Since HSCs are proposed to migrate from the AGM region to the fetal liver at around E11-E12, most likely via the circulation, migratory aspects are important in the regulation of HSCs during midgestation development. Expression changes in cell adhesion molecules induced by IL-1 could be determined again by FACS analysis. The functional properties could be assayed in cell

migration assays as routinely used by others. Furthermore, it is not clear whether IL-1 act directly on AGM HSCs or whether it acts on other cell types that induce changes in AGM HSCs. As the IL-1RI is expressed almost exclusively on $CD45^+$ (hematopoietic) cells in the AGM and liver, it is highly likely that IL-1 exerts its effects directly on hematopoietic cells and not through an intermediate cell type. Studies with adult bone marrow HSCs showed that IL-1 usually synergizes with other growth factors to induce proliferation of these cells. Whether IL-1 also cooperates with other cytokines or growth factors in the midgestation AGM and liver remains to be investigated.

The role of TAB2 in Interleukin-1 signaling and development.

The questions that remain are what role does TAB2 play in IL-1 signaling in these AGM cells and what is the function of IL-1 and TAB2 during development? The first reports on TAB2 proposed a model in which TAB2 functions as an adapter protein, linking TAK1 to the IL-1R and thereby mediating IL-1 signaling[44,45] (Figure 1A). However, these initial studies performed by Matsumoto and co-workers overexpressed TAK1 and TAB proteins in a non-IL-1R expressing cell line co-transfected with the *Il-1r* gene. In retrospect, careful examination of the biochemical method used to detect TAB2 and TAK1 translocation from the plasma membrane to the cytosol, reveals that the so-called plasma membrane fraction also contains the nuclear fraction from these cells. Therefore, no conclusions on the localization of TAK1 and TAB2 can be drawn from these experiments. Moreover, the proposed crucial function of TAB2 in IL-1 signaling was based on the observation that a truncated TAB2 protein blocks NFκB and JNK activation upon IL-1 stimulation. The puzzling fact that mere overexpression of full length TAB2 resulted in *Nfκb* promoter activity and JNK kinase activity in the absence of IL-1 stimulation, remained uninvestigated in these studies.

Consistent with the possibility that TAB2 might be localized in the nucleus, a recent report showed by immunofluoresence studies that indeed TAB2 in neuronal cells is localized in the nucleus and functions as a repressor protein of p50 NFκB-mediated gene expression[5] (Figure 1B). The recent report of *Tab2* deficient mice may support this model, as *Tab2* deficient cells were found to normally activate *NFκB* promoter activity and JNK kinase activity upon IL-1 stimulation[36]. Thus, TAB2 may not be required for the early steps of IL-1 signaling. Alternatively, TAB2 may play a role proximal to the IL-1 receptor as an adapter, but is functional redundant with the highly homologous TAB3 protein.

TAB2: a role in apoptosis

Concerning the hematopoietic system, the phenotype of the *Tab2* deficient mice did reveal that TAB2 is required to prevent fetal liver apoptosis during mouse development and that TAK1 and TAB1 phosphorylation (and likely activation) is dependent on TAB2 expression[36]. Together with the studies from Baek et al., these studies provide important clues about the function of TAB2, namely that TAB2 is involved in apoptosis regulation as well as NFκB activity regulation[5 36]. In the studies presented here, we observed nuclear localization of TAB2 in hematopoietic and 3T3 fibroblast cells. However, low levels of TAB2 were also observed in the cytoplasm. This raises the possibility that (in some cell types) TAB2 might not only be modulating NFκB activity in the nucleus, but that TAB2 also fulfils additional roles in the cytoplasm. In this regard it is interesting that TAB2 interacts with TRAF6, another regulator of IL-1 and TNFα signaling, but also an inducer of

apoptosis[3,44]. It has been suggested that the composition of these TRAF proteins in multi-protein aggregates in the close proximity to their receptors determines cell fate, resulting either in cell viability or cell death[3]. Baud et al. showed that clustering of TRAF2 or TRAF6 proteins could induce NFκB and JNK activation resulting in subsequent target gene induction in the absence of IL-1 or TNFα stimulation[6]. Therefore, one could speculate that the reported effect of NFκB and JNK activation upon overexpression of TAB2 [44,45] might be due to inappropriate cellular localization and/or enhanced binding of TAB2 to TRAF proteins resulting in clustering and thereby activating the signaling cascade in the absence of IL-1 stimulation. However, this possibility remains speculative until the appropriate experiments are performed.

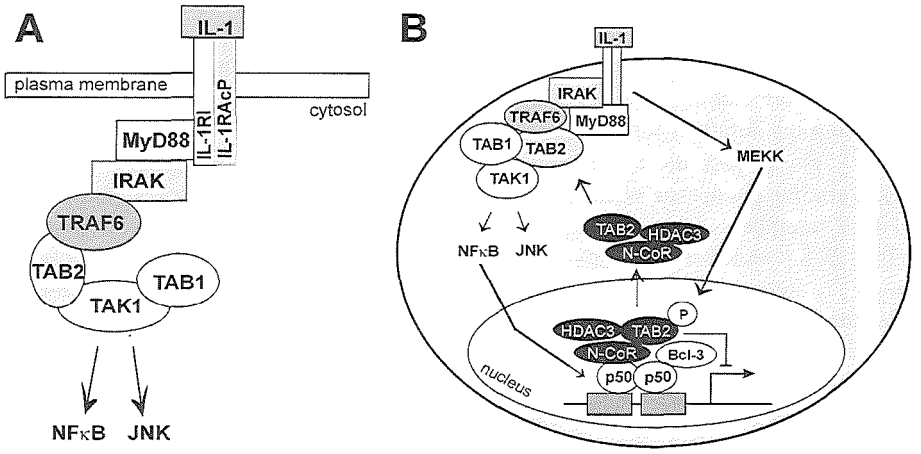

Figure 1. Models for the role of TAB2 in IL-1 signaling
(A) Model as proposed by Takaesu et al. implicates TAB2 as an adapter protein in the proximal IL-1 signaling cascade [44]. Binding of IL-1 to the IL-1 receptor results in recruitment of the adapter protein MyD88, the kinase IRAK and the adapter protein TRAF6. TRAF6 will bind to TAB2, and allows the TAK1-TAB1 complex to be brought in close proximity of the receptor. Activation of TAK1 results in NFκB and JNK activation and further signaling. (B) Model as proposed by Baek et al. for TAB2 function[5]. Without IL-1 stimulation, TAB2 is localized in the nucleus in a complex with NFκB subunits (p50) at the promoters of NFκB target genes. The presence of TAB2 mediates the repression of these NFκB target genes. Upon binding of IL-1 to the IL-1 receptor, MEKK is activated, translocates to the nucleus and phosphorylates TAB2. Subsequently, TAB2 is translocated from the nucleus to the cytoplasm, thereby relieving the repression of the NFκB target genes. Baek et al. additionally suggested that TAB2 in the cytosol could function to activate NFκB and JNK signaling via the interaction with TAK1 proximal of the IL-1 receptor.

Also, TAK1 and TAB1 have been shown to induce apoptosis *in vivo*. Studies in *Xenopus* show that overexpression of TAK1 and TAB1 results in apoptosis and lethality during development. Overexpression of the anti-apoptotic Bcl-2 gene was required in these studies to reveal that TAK1 overexpression leads to the ventralization of the embryos at the expensive of neural tissue development[39]. Moreover, it was shown that TAK1 and TAB1

function downstream of the BMP receptor in Xenopus and are linked to the BMP receptor via XIAP[50], which is known as an inhibitor of apoptosis. This raises the intriguing possibility that TAK1/TAB1 overexpression induces apoptosis by sequestering XIAP (or related IAP proteins). Thus, one could speculate that by regulating the availability and the local intracellular concentrations of TRAF (and IAP) proteins, the balance between cellular viability and apoptosis might be regulated by TAK1 and TAB proteins. Therefore, it would be interesting to examine, in the mouse system, in more detail the intracellular localization of endogenous and overexpressed TAB2 full length and truncated proteins and to determine whether these proteins interact with known regulators of apoptosis. Such transgenic studies could reveal whether the level of TAB2 expression is important in regulating the balance between cell viability and apoptosis in a physiological context.

In addition to acting directly on apoptosis regulators, TAB2 may regulate apoptosis and other cellular functions via the interaction with NFκB proteins to modulate NFκB activity. Several members of the NFκB family have been implicated as important regulators of cell viability and apoptosis[12,23]. Interestingly, embryonic lethality has been observed in mice deficient for the NFκB member *RelA* or the NFκB regulator *IκB kinase 2* (*Ikk-2*) [7,26]. These mice die due to fetal liver apoptosis around E15 (*RelA*) or E12.5-E14 (*Ikk-2*), a similar phenotype observed for *Tab2* deficient mice [36]. Moreover, it was shown that NFκB members p65 and c-rel regulate gene expression of anti-apoptotic genes, including *Bcl-2* and *Bcl-x_L*.[8,18]. *Bcl-x_L* deficient mice die around E13 due to fetal liver apoptosis [31]. Interestingly, the expression pattern of Bcl-x_L and some NFκB members includes the fetal liver and adult hematopoietic tissues, and is similar to that of TAB2[17,24].

The proposed function by Baek et al. for TAB2 as a regulator of NFκB activity is so far consistent with reported studies that TAB2 is involved in apoptosis, which could be regulated via NFκB, as well as with the observations that TAB2 does not play a crucial role in proximal IL-1 receptor signaling. However, as it was reported that the complex of TAB2 and NFκB at the promoters of certain target genes is disrupted upon IL-1 stimulation, TAB2 can still be regarded as a regulator of IL-1 signaling although at a different level in the signaling cascade than originally reported.

Is TAB2 involved in other signaling pathway?

Based on the phenotypes of the IL-1 signaling component deficient mice in comparison with the *Tab2* deficient mice, it is unlikely that the function of TAB2 is restricted to IL-1 signaling. Several mouse models have been generated that lack expression of the ligand IL-1, the IL-1 receptor or components of the IL-1 signaling cascade, including MyD88 and IRAK-4 [1,16,20,43]. In addition, also mice deficient for the closely related *Il-18* receptor have been generated [21]. These mice all display a similar phenotype. They do not show any developmental defects, are unresponsive to IL-1 (or IL-18) stimulation and display defects within the immune system, including defects in B-cell activation and fever induction upon infection [1,16,20,43,21] . In contrast, the *Tab2* deficient mice display a rather severe phenotype, with embryonic lethality around E12.5[36]. Moreover, approximately 90% of the heterozygous *Tab2* deficient mice die within two weeks after birth, indicating that the dose of TAB2 is also important [36]. Thus, these dramatic phenotypic differences indicate that

the function of TAB2 is not solely restricted to IL-1 signaling. Furthermore, by immunohistochemistry studies for TAB2 and IL-1RI in the midgestation mouse embryo we observed extensive, but not a complete, overlap in the expression of these proteins. Therefore, it seems likely that TAB2 fulfils a more general role in regulating some cellular processes.

Apoptosis: a role in HSC development?

Besides the studies on the expression of TAB2 and the role of IL-1 on HSC regulation during mouse development, we also studied the effects of Bcl-2 overexpression on the hematopoietic compartment in the adult mouse and during development. Bcl-2 protein family members clearly play an important role in the regulation of apoptosis[2]. As described in chapter 5, *Bcl-2* overexpression was directed by the *Ly6E* (Sca-1) promoter in transgenic mice. As expected from overexpressing an anti-apoptosis regulator, we observed increased cell viability under stressful conditions *in vivo*, by total body irradiation studies, and *in vitro* by growth factor withdrawal in cell culture. However, also in steady-state conditions, without inducing cellular stress, we observed increased numbers of c-kit$^+$ cells in the adult bone marrow. Moreover, upon transplantation we observed a higher HSC repopulation capacity in bone marrow cells of Bcl-2 overexpressing mice. Interestingly, also in the E11 AGM and liver we observed higher HSC repopulation capacity, suggesting that from the earliest developmental stages onwards HSCs might be normally prone to apoptosis. Whether this process of apoptosis already occurs *in vivo* during the development of the AGM or during the process of transplantation and/or homing to the bone marrow of these AGM HSCs is currently unclear. To determine whether apoptosis plays a role during the transplantation and/or homing process marked AGM HSCs (i.e. *Ly6A-GFP* derived HSCs) could be used for transplantation and thereafter the viability and homing efficiency of these cells could be determined. Taken together, further investigations are required to examine in more detail the expression and function of apoptosis regulators, including TAB2, within the hematopoietic compartment in the AGM and fetal liver.

Conclusions

The aim of the investigations presented here was to search for novel regulators of hematopoietic stem cells in the midgestation AGM region of the mouse embryo. By means of differential screening we found several differentially expressed genes in the AGM region at the time of HSC emergence. One of these genes, the novel mouse gene *Tab2*, was shown to be expressed in a spatio-temporal manner consistent with known HSC activity. Although it has not been elucidated how TAB2 is involved in HSC regulation, we were able to reveal that the IL-1 signaling pathway, in which TAB2 has been proposed to play a role, does affect the earliest definitive HSCs during mouse development. Moreover, we were able to reveal a possible role for apoptosis in these cells by means of *Bcl-2* overexpression studies. Thus, while there are many questions that still need to be answered, these studies have led to increased insight into the numerous and highly controlled regulatory mechanisms that lead to the balanced emergence, expansion and maintenance of HSCs during mouse development.

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Summary

The hematopoietic system fulfills important roles for the viability of whole organisms from embryonic to adult stages of life. For example, the erythroid branch of the hematopoietic hierarchy supplies oxygen to tissues and organs, while the lymphoid branch plays a crucial role protecting the organism against invading pathogens. These functions are carried out by the mature, differentiated cells of the hematopoietic system. Mature hematopoietic cells are produced through a complex differentiation hierarchy founded by hematopoietic stem cells (HSCs). During embryonic development HSCs are first generated in close association with one of the major blood vessels in the embryo, the dorsal aorta. Together with the aorta, the surrounding region, comprised of the primordial kidney and reproductive anlagen, is referred to as the aorta-gonad-mesonephros region or AGM. During mouse development, HSCs can be found from embryonic day 10.5 onwards in the AGM region and one day later in the fetal liver, where they remain until neonatal stages. Several regulators that play a role, in either the emergence and/or regulation of HSCs in the embryo and the adult, have been identified over the years. Despite this knowledge and the fact that the insight in the regulatory processes are rapidly increasing, there are still gaps in the understanding of the molecular events leading to the emergence of HSCs.

To gain further insight into the genes that play a role in mouse HSCs and the cells that surround them in the AGM, we set out to identify genes that were differentially expressed (or in other words "turned on" or "turned off"), in the AGM region at the time of HSC emergence. By performing a screening for such genes, we identified several genes that were differentially expressed, among which were two novel mouse genes. These novel mouse genes were highly homologous to known human genes, suggesting they might have a similar function in human and mice. One of these genes, *TAB2*, had been suggested to play a role in interleukin-1 (IL-1) signaling. As *TAB2* is a novel gene, little information is available about its expression pattern or its role in normal physiology. Therefore, we investigated the expression pattern of TAB2 in the adult mouse and during embryonic development. Besides expression in hematopoietic organs, TAB2 is also expressed in neural tissues, throughout embryonic and adult stages. Since others have shown that IL-1 is a regulator of adult bone marrow HSCs, and TAB2 is thought to function in the IL-1 signaling pathway, we examined whether IL-1 also functions as a HSC regulator during development. Our studies show that IL-1 is a potent HSC regulator in the AGM and fetal liver. In related studies to investigate the role of cell death in the regulation of HSCs in the adult and the embryo, we used a transgenic mouse model in which the cell survival gene *Bcl-2* is overexpressed within HSCs. Our results indicate that indeed cell death and survival plays an important role in the hematopoietic system and particularly in HSCs throughout development.

Altogether, these studies have resulted in novel insights into the regulation of HSCs in the midgestation mouse embryo. Both a novel gene, *Tab2*, and a known hematopoietic regulator, IL-1, have been implicated for the first time as possible regulators of HSCs in the AGM and fetal liver. TAB2, as a mediator of the pro-inflammatory regulator IL-1, is expressed in both neural and hematopoietic tissues. Therefore TAB2 is an interesting candidate for further studies concerning hematopoietic and neural regulation during embryonic development, adult tissue homeostasis and inflammation-mediated diseases.

Samenvatting voor iedereen

Het hematopoietische (bloed) systeem vervult een aantal verschillende belangrijke taken zowel gedurende de embryonale ontwikkeling als in volwassen dieren. Zo zorgen bijvoorbeeld de rode bloedcellen (erythrocyten) voor zuurstoftransport naar de verschillende organen en weefsels, en de witte bloedcellen (lymfocyten) spelen een rol in het immuun systeem dat ons lichaam beschermt tegen allerlei ziekteverwekkers en lichaamsvreemde stoffen. Deze functies van het hematopoietische systeem worden uitgevoerd door rijpe/ gedifferentieerde bloedcellen, waarvan de meeste bloedceltypes een relatief korte levensduur hebben. Een continue aanmaak van deze cellen is dan ook noodzakelijk om het hematopoietische systeem in stand te houden. Nieuwe gedifferentieerde cellen worden gevormd via een complex netwerk van hematopoietische voorlopercellen die op hun beurt weer afstammen van de hematopoietische stamcellen.

In de volwassen mens en muis bevinden de hematopoietische stamcellen zich in het beenmerg. Echter, tijdens de embryonale ontwikkeling van de mens en muis ontstaan deze hematopoietische stamcellen in de nabijheid van een van de grootste bloedvaten van het embryo, de dorsale aorta. In het gebied tussen de voor-en achterpoten van de muis wordt de dorsale aorta omringd door weefsels die later in de embryonale ontwikkeling de nier en de geslachtsorganen gaan vormen. Deze regio wordt aangeduid als de aorta-gonade-mesonephros regio ofwel AGM regio. Halverwege de ontwikkeling van de muis (tussen dag 10 en 11) ontstaan hematopoietische stamcellen in de AGM regio in de nabijheid van de dorsale aorta. Vlak na hun ontstaan, verlaten de hematopoietische stamcellen de AGM regio en kunnen ze gevonden worden in de foetale lever. De hematopoietische stamcellen blijven tot vlak voor de geboorte in de lever waarna ze naar het beenmerg gaan om van hieruit de verschillende gedifferentieerde hematopoietische cellen te gaan produceren. Sinds lange tijd wordt er onderzoek gedaan om een beter inzicht te krijgen in het ontstaan en de regulatie van de hematopoietische stamcellen gedurende de embryonale ontwikkeling. Ondanks het feit dat een aantal belangrijke spelers in deze processen zijn geïdentificeerd en gekarakteriseerd, is er nog geen volledig inzicht in de processen die zich afspelen in het embryo met betrekking tot het ontstaan en de regulatie van hematopoietische stamcellen.

Om de inzichten in de regulatie en ontstaanswijze van hematopoietische stamcellen in de AGM regio te vergroten, hebben we onderzocht welke genen differentieel tot expressie komen (ofwel welke genen "aangezet" en welke genen "uitgezet" worden) op het moment dat hematopoietische stamcellen ontstaan in de AGM regio. Zoals beschreven in hoofdstuk 2, hebben we een aantal genen gevonden die differentieel tot expressie komen, waaronder twee genen die in de muis nog niet eerder waren geïdentificeerd. Deze twee genen waren echter in de mens al wel eerder beschreven en onderzoek wees uit dat deze genen tussen de mens en de muis nagenoeg identiek zijn. Dit zou erop kunnen duiden dat ook de functie van deze genen in de muis en mens vergelijkbaar is.

Naar een van deze twee genen, namelijk *Tab2*, is verder onderzoek gedaan. De eerste studie naar de functie van TAB2 is gepubliceerd in 2000 door Takaesu en collega's. Deze studie suggereert dat TAB2 een rol speelt in de interleukine-1 (IL-1) signaleringsroute. IL-1 is een cytokine dat een belangrijke rol speelt in de regulatie van ontstekingsreacties en waarvan bekend is dat het hematopoietische cellen, waaronder stamcellen, kan reguleren en activeren. Aangezien *TAB2* een relatief nieuw gen is en er

daarom dus ook weinig over bekend is, hebben we eerst het expressie patroon in de volwassen muis en in het embryo onderzocht, zoals beschreven in hoofdstuk 3. TAB2 komt tot expressie in verschillende hematopoietische organen, zoals cellen in het beenmerg, de milt en de thymus, maar ook in verschillende neurale weefsels, waaronder cellen in de hersenen en het ruggemerg.

Naast het onderzoek naar het expressiepatroon van TAB2 hebben we ook getracht om de functie van TAB2 en IL-1 te achterhalen. In de volwassen mens en muis zijn al veel studies gedaan naar de rol die IL-1 speelt in de regulatie van hematopoietische cellen. Er is echter weinig bekend over de rol van IL-1 tijdens de embryonale ontwikkeling. Aangezien TAB2 een rol speelt in de IL-1 signaleringsroute, hebben wij onderzocht of IL-1 ook een rol speelt in de regulatie van hematopoietische cellen in muizenembryos. Zoals beschreven in hoofdstuk 4 laat ons onderzoek zien dat IL-1 een regulator is van hematopoietische stamcellen vanaf het moment dat deze cellen in het embryo ontstaan. Op dit moment is het nog niet helemaal duidelijk hoe IL-1 zijn regulatoire functie op hematopoietische stamcellen uitoefent en tevens is het niet duidelijk welke rol TAB2 speelt in de signaleringsroute van IL-1. Verder onderzoek zal hierover uitsluitsel moeten geven. Op basis van bevindingen van anderen, zijn er enkele mogelijkheden voor de rol van IL-1 in de regulatie van hematopoietische cellen. De meest waarschijnlijke mogelijkheden zijn dat IL-1 de migratie van hematopoietische stamcellen van de AGM regio naar de foetale lever reguleert of dat IL-1 een rol speelt in de instructie van cellen tussen de keuze op leven en dood van deze cellen in het embryo.

Om beter inzicht te krijgen in de rol die gereguleerde celdood speelt in de ontwikkeling van het hematopoietische systeem, is er een muizenmodel gemaakt door middel van transgenese. De beslissing tussen leven en doodgaan van de cel wordt in belangrijke mate bepaald door de balans tussen het expressieniveau van een aantal genen, waarvan enkele celdood bevorderen en andere genen juist de levensvatbaarheid van cellen vergroten. Door middel van signalen van buitenaf wordt de cel beïnvloedt en zal de balans tussen deze genen gereguleerd worden. In het muizenmodel waarvan wij gebruik hebben gemaakt is de regulator Bcl-2 tot overexpressie gebracht in het hematopoietische systeem. Anderen hadden al aangetoond dat hoge expressieniveaus van Bcl-2 de levensvatbaarheid van verschillende celtypes kan vergroten. Uit onze studies, die beschreven zijn in hoofdstuk 5, blijkt dat dit ook het geval is voor hematopoietische stamcellen in het embryo. Tevens duiden de resultaten erop dat regulatie van leven en dood van hematopoietische stamcellen in het embryo een belangrijke rol speelt in de normale ontwikkeling van het hematopoietische systeem.

Samengevat hebben de studies die in dit proefschrift beschreven staan nieuwe inzichten opgeleverd met betrekking tot de regulatie van hematopoietische stamcellen gedurende de embryonale ontwikkeling van de muis. Dit is de eerste studie die de rol beschrijft van TAB2 en van IL-1 in de regulatie van hematopoietische stamcellen in de AGM regio en de foetale lever gedurende de embryonale ontwikkeling van de muis. Verder onderzoek naar TAB2 zal zowel in het hematopoietische als in het neurale systeem belangrijke inzichten opleveren met betrekking tot de embryonale ontwikkeling, homeostase in volwassenen en ontstekingsgerelateerde processen.

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Liefs,

Claudia

Curriculum Vitae

Claudia Orelio werd geboren op 7 mei 1976 te Zaandam (gemeente Zaanstad). In 1994 behaalde zij haar VWO (Atheneum) diploma aan het Hendrik Pierson College te Zetten en begon in september 1994 met de studie Biologie aan de Universiteit Utrecht. In 1995, na behalen van het propedautisch examen Biologie, vervolgde zij haar studie met de bovenbouwstudie Fundamentale Biomedische Wetenschappen. Tijdens haar studie zijn twee stages doorlopen, waarvan de eerste werd uitgevoerd onder begeleiding van Drs. J.Veltmaat en Dr. L.H.K.Defize aan het Hubrecht Laboratorium te Utrecht. De tweede stage werd uitgevoerd onder begeleiding van Dr. K.Reedquist en Prof. J.L.Bos bij de vakgroep Fysiologische Chemie, Universiteit Utrecht. De scriptie werd aansluitend aan het einde van de eerste stage geschreven onder begeleiding van Dr. L.H.K.Defize. Op 31 augustus 1999 werd het doctoraalexamen Fundamentele Biomedische Wetenschappen behaald.

Vanaf september 1999 werkte ze als promovendus bij de vakgroep Celbiologie en Genetica, Erasmus Universiteit Rotterdam, onder begeleiding van Prof. E. Dzierzak. Dit promotie onderzoek had tot doel meer inzicht te krijgen in de moleculaire en genetische processen die een rol spelen gedurende het onstaan en verdere regulatie van hematopoietische stamcellen tijdens de embryonale ontwikkeling van de muis. Vanaf september 2003 zal het onderzoek naar de regulatie van hematopoietische stamcellen gedurende de embryonale ontwikkeling in de groep van Prof. E.Dzierzak als Postdoc worden voortgezet.

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