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The regulation of hematopoiesis by stromal cells

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Index

Index	2
Summary.....	4
Zusammenfassung.....	4
Abbreviations	6
Chapter 1: Introduction to hematopoiesis	8
1.1 Hematopoietic overview	9
1.2 Hematopoietic development.....	9
1.3 The hematopoietic stem cell niche	14
1.4 Dysregulation of the niche may cause hematopoietic abnormalities	20
1.5 Summary and conclusions	21
1.6 Aim of thesis	22
1.7 Acknowledgements	24
1.8 References.....	24
Chapter 2: Previous work	38
2.1 Studies prior to my arrival	39
2.2 References.....	41
Chapter 3: Identifying new regulators of hematopoiesis	42
3.1 Yeast 2 hybrid and Mouse Atlas arrays.....	43
3.2 Old array data results.....	45
3.3 Yeast 2 hybrid results.....	46
3.5 Discussion.....	50
3.4 Materials and methods.....	52
Chapter 4: New gene expression array, Affymetrix	56
4.1 Background.....	57
4.2 Results	57
4.3 Discussion.....	59
4.4 <i>Sfrp1</i>	61
4.5 Materials and methods.....	63
4.6 References.....	64
Chapter 5: Secreted frizzled-related protein 1 (<i>Sfrp1</i>) extrinsically regulates cycling activity and maintenance of hematopoietic stem cells	67
5.1 Summary.....	68
5.2 Introduction	68
5.3 Results	71
5.4 Discussion.....	85
5.5 Experimental procedures	88
5.6 Acknowledgements	92
5.7 Supplementary data: Renström et al. 2009.....	93
5.8 References.....	102

Chapter 6: Characterization of the mature hematopoietic cell populations in <i>Sfrp1</i>^{-/-} mice.....	106
6.1 Introduction	107
6.2 Results	108
6.3 Materials and methods	113
6.4 Discussion.....	114
6.5 References.....	116
Chapter 7: Efficient Hematopoietic Differentiation of Human Embryonic Stem Cells on Stromal Cells Derived from Hematopoietic Niches	118
7.1 Summary.....	119
7.2 Introduction	119
7.3 Results	121
7.4 Discussion.....	138
7.5 Experimental procedures	142
7.6 Acknowledgements	146
7.7 Supplemental data	147
7.8 References.....	164
Chapter 8: Conclusion and future aspects.....	169
8.1 Conclusion	170
8.2 Human embryonic stem cell project	170
8.3 <i>Sfrp1</i> as a modulator of hematopoiesis.....	171
8.4 Future aspects	175
8.5 Acknowledgements.....	177
8.6 References.....	178

Summary

Using cell lines derived from the aorta-gonads mesonephros (AGM)-region in the mouse embryo it was possible to identify, by using gene expression arrays, *Sfrp1*, *Tgfb1* and *Tgfb3* as novel regulators of hematopoiesis. Mice deficient in *Sfrp1* suffer from a wide range of significant hematopoietic abnormalities, even though none of them are lethal. Abnormalities included: increased cell numbers in peripheral blood, increased numbers of phenotypical hematopoietic stem cells (HSC) in the bone marrow, more HSCs in the G0/G1 phase of the cell cycle, increased production by hematopoietic progenitor cells, and decreased amount of β -catenin. *Sfrp1* deficient mice also displayed a loss of HSC and multipotent progenitors (MPP) in serial bone marrow transplantations but no significant loss of function of HSC in limiting dilution transplantations. Furthermore, *Tgfb1* and *Tgfb3* were shown to enhance human embryonic stem cell (hESC) hematopoietic differentiation and can further stimulate this process when added to cultures. In short, this thesis highlights novel ways for the microenvironment to regulate hematopoiesis.

Zusammenfassung

Durch Genexpressionsarrays von Zelllinien aus der AGM-Region von Mausembryonen war es möglich *Sfrp1*, *Tgfb1* und *Tgfb3* als neue Hämatopoeseregulatoren zu identifizieren. *Sfrp1*-defiziente Mäuse weisen eine Vielzahl hämatopoetischer Anomalien auf, welche jedoch nicht letal verlaufen. Zu diesen Anomalien gehören erhöhte Zellzahlen im peripheren Blut, erhöhte Anzahl phänotypischer HSC im Knochenmark, welche sich verstärkt in der G0/G1-Phase des Zellzyklus befinden, erhöhte Produktion hämatopoetischer Vorläuferzellen, verminderte Mengen an β -catenin und Verlust von HSC und MPP in seriellen Knochenmarktransplantationen. Es wurde jedoch kein bedeutender Funktionsverlust von HSC in „limiting dilution“ Transplantationen gesehen. Für *Tgfb1* und *Tgfb3* konnte gezeigt werden, dass sie die hämatopoetische Differenzierung von hESC fördern und diese bei Zugabe zu Kulturen weiter anregen. Zusammenfassend lässt sich sagen, dass diese Arbeit neue Wege der Hämatopoeseregulation durch die Mikroumgebung im Knochenmark behandelt.

Ich erkläre an Eides statt, dass die vorliegende Dissertation in allen Teilen von mir selbständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben wordensind.

Abbreviations

1° = primary transplantation

2° = secondary transplantation

AD = activating domain

AGM = aorta-gonads mesonephros

AM = aorta/mesenchyme

APC = Alyophycocyanin

BM = bone marrow

BrdU = Bromodeoxyuridine

cDNA = coding DNA

CFC = Colony forming cell

CLP = common lymphoid progenitor

CM = conditioned medium

CMP = common myeloid progenitor

DB = DNA binding domain

DMEM = Dulbecco's Modified Eagle Medium

DNA = DeoxyriboNucleic Acid

E = days after fertilization

EB = embryoid body

EL = embryonic liver

ELISA = Enzyme-linked immunosorbent assay

ESC = embryonic stem cell

FACS = Fluorescent-Activated Cell Sorting

FCS = Fetal calf serum

FELASA = Federation of European Laboratory Animal Science Associations

FITC = fluorescein isothiocyanate

Fzd = Frizzled

g = gram

GAD = pGADT7 Activation domain cloning vector

GBK = pGBKT7 DNA binding cloning vector

GI = gastrointestinal tract

GMP = granulocyte-monocyte progenitors

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

His = Histidin

HSC = Hematopoietic stem cell

kb = kilo base pairs

kDa = kilo Dalton

l = litre

Leu = Leucin

Lin = lineage

LSK = Lineage negative, Sca1 positive and Kit positive cells

LT-HSC = long term repopulating hematopoietic stem cell

m = milli

M = molar (mol/dm^3)

MEP = megakaryocyte-erythroid progenitors

MPP = Multipotent progenitor

mRNA = messenger RNA

N = sample size

PB = peripheral blood

PBS = phosphate buffered saline

PCR = polymerase chain reaction

PE = phycoerythrin

PIPES = 1,4 Piperazine bis (2-ethanosulfonic acid)

RNA = RiboNucleic Acid

RT-PCR = reverse transcriptase PCR

shRNA = short hairpin RNA

SPF = specific pathogen free

ST-HSC = short term repopulating hematopoietic stem cell

Trp = tryptophane

μ = micro

UG = urogenital ridges

Wnt = Wingless (Wg) and Int, combined to Wnt, usually referring to the Wnt signalling pathway

WT = wild type

Chapter 1: Introduction to hematopoiesis

How the Niche Regulates Hematopoietic Stem Cells

Chem Biol Interact

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1.1 Hematopoietic overview

The hematopoietic system is what makes up all of the blood cells in the body, be it white leuko- and lymphocytes or red blood cells. These cells are required for a multitude of tasks, such as protection from infections, removal of damaged tissue, but also to transport vital molecules such as oxygen to where it is needed throughout the body. Most of these hematopoietic cells have a limited life span and since there is a continuous need of cells to perform these tasks throughout life, a system to continuously replenish their numbers is required for the organism to survive.

At the centre of this complex machinery sits the hematopoietic stem cell (HSC), which, in the adult mammalian, primarily resides in the bone marrow (BM). This cell is unique among hematopoietic cells by having the potential to give rise to all the cells in the hematopoietic system and at the same time be capable of self-renewal. Self-renewal is an important cellular event, which means a cell can divide while maintaining at least one daughter cell in the undifferentiated stem cell-like state. Due to this capacity, HSC can create an entire hematopoietic system from just a single cell and maintain clonal hematopoiesis for the lifetime of the individual (Ema et al., 2006). Since HSC reside in the bone marrow, the current idea is that the bone marrow microenvironment, or so-called niche, plays an important role in the regulation of self-renewal and differentiation of HSC. The microenvironment is the collective concept for the different types of cells and structures surrounding the bone, which regulates the fate of hematopoietic cells through direct or indirect means, facilitating a stable generation of all the blood cells needed in a steady state situation. But, the microenvironment also adapts in times of hematopoietic stress. A failure to maintain a strict regulation of the hematopoietic cells can lead to a variety of malignancies such as different types of leukaemia, the most common forms of cancer in humans.

1.2 Hematopoietic development

1.2.1 Embryonic hematopoiesis

In the mouse, the earliest hematopoietic cells found are the primitive, nucleus bearing erythrocytes in the yolk sac at embryonic day 7.5 (E7.5) (Moore and Metcalf, 1970). At this early stage of development the primitive hematopoietic cells are organized in “blood islands”, which also contain blood vessels. These blood vessels are believed

to originate from primitive hemangioblasts, which are capable of producing both hematopoietic and endothelial cells (Palis and Yoder, 2001). The molecular mechanisms of blood island formation are, as yet, not elucidated entirely. The basic helix-loop-helix transcription factor *Scl* is essential for both primitive and later definitive hematopoiesis, and deficiency in either *Gata1* or *Gata2* also impairs formation of functional blood islands (reviewed by (Ferkowicz and Yoder, 2005)). Since vascularisation is important for hemangioblasts to develop, molecules involved in vascular development, such as *Kdr* (also known as *Flk1*, *Vegfr2*), are required in the developing embryo for this early hematopoietic differentiation to occur (Shalaby et al., 1995). After the emergence of primitive hematopoiesis in the yolk sac, a second wave of definitive hematopoiesis can be detected around the stage where circulation starts (E8.25). There is now evidence supporting the notion that precursors of definitive cells emerge in the yolk sac, which subsequently seed the embryo proper (Lux et al., 2008). The first definitive HSC that can regenerate hematopoiesis in adult recipients can be detected in the aorta-gonads-mesonephros (AGM) region, around day E10.5 (Medvinsky and Dzierzak, 1996). The role of core-binding transcription factor *Runx1* in the emergence of the definitive HSC has been firmly established (North et al., 1999). These first HSCs appear in the aorta sub region of the ventral AGM (de Bruijn et al., 2000), possibly as a product of hemogenic endothelium (Jaffredo et al., 2005a; Jaffredo et al., 2005b; North et al., 2009; Sugiyama et al., 2003). Only slightly later in development, HSC are also detectable in the placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005), circulation and yolk sac of the embryo and subsequently the number of HSC in the fetal liver starts to rise (Kumaravelu et al., 2002). Besides *Runx1*, other transcription factors may play additional roles in the development of definitive hematopoiesis. For instance, the mixed leukemia gene (*Mll*) (Ernst et al., 2004), *Meis1* (Azcoitia et al., 2005), and the Notch pathway factor *Rbpj* (Robert-Moreno et al., 2005), all frequently found to be involved in dysregulation of adult leukemias, may be as well important. Until birth, the fetal liver is the main hematopoietic organ. From the fetal liver, HSC migrate and colonize the bone marrow (BM) just prior to birth and from here onwards adult hematopoiesis in the bone marrow as its primary site of activity.

Recently, it was demonstrated that besides the transition from primitive to definitive hematopoiesis, a second event is the switch from fetal to adult hematopoiesis: the fetal program (high proliferative capacity) remained until about 3-4 weeks after birth, and then switched to the adult program where HSC are quiescent and mainly in

G0/G1 of cell cycle (Bowie et al., 2006). Another study revealed a possible candidate to identify the switch from embryonic to adult hematopoiesis; the transcriptional regulator *Sox17*, which is clearly expressed in fetal HSC but not in adult HSC more than 4 weeks after birth in mice (Kim et al., 2007). Indeed, a conditional knockout of *Sox17* has severe effects on fetal, actively cycling HSC, but does not seem to affect adult, quiescent HSC (Kim et al., 2007).

1.2.3 The adult mouse hematopoietic hierarchy

After the switch to the adult hematopoietic program, the HSC mainly resides in the bone marrow. Since the number of HSC is relatively small to the vast number of cells needed each day in the living organism, the entire proliferation and hematopoietic maturation process is tightly regulated. To understand these mechanisms, and to understand how these mechanisms can be disturbed by environmental cues, the precise description of all cell types involved and their lineage relationships facilitates the exact pinpointing of changes in the regulation of their proliferation and differentiation.

The adult definitive hematopoietic hierarchy consists of a chain of progressively maturing hematopoietic cells culminating with the mature red and white blood cells mainly found in the circulation, but also in other hematopoietic tissues: spleen, thymus, and lymph nodes. In the mouse, this hierarchy has been described in such detail that single stem cells can be isolated and their behaviour studied. Thus, the hematopoietic system is an excellent model to study changes in stem cell regulation caused by environmental cues.

The characterization and isolation of different hematopoietic subsets is today usually performed using fluorescent-activated cell sorting (FACS) which relies on attaching fluorescent probes (labeled antibodies) to certain known surface markers expressed by cells. A cell expressing the marker will then get these fluorescently labeled antibodies bound to it, which in turn emits light of different wavelengths when excited by a laser, which can then be detected by the flow cytometer machine, indicating that a cell is positive for the selected marker. In order to single out the HSCs among all others one has to look at a combination of antibodies, which both exclude and include the true HSC population, fortunately the FACS machines of today are capable of simultaneously detecting a range of different fluorescent probes. A summary of markers used to distinguish the earliest HSC from their more mature

counterparts is given in Table 1. HSC have been phenotypically described by the absence of surface expression of lineage markers (Lin⁻) and the presence of both Ly-6A/E (Sca-1) and Kit (Ikuta and Weissman, 1992). Further improvements of this phenotype facilitate the isolation of single true HSC transplanted. Among those are the recognition that HSC do not express Cd34 (Osawa et al., 1996), Flk2/Flt3 (Christensen and Weissman, 2001), or Cd48 (Slamf2), but do express Cd150 (Slamf1) (Kiel et al., 2005) (Figure 1A), and the EPC receptor (Balazs et al., 2006; Dykstra et al., 2007; Kent et al., 2009). These HSC divide and mature into different cell subsets which first become positive for Cd34, Flk2 and Cd48, and after a number of further differentiation steps then form the two basic mature blood cell types: myeloid and lymphoid cells. The differentiation of these two major lineages is thought to separate quite early in the maturation hierarchy at the multipotent progenitor (MPP) stage into interleukin 7 receptor (Il7r)-expressing common lymphoid progenitors (CLP) (Kondo et al., 1997) and common myeloid progenitors (CMP) (Akashi et al., 2000) (reviewed in (Lai and Kondo, 2008)). These CMP will then further differentiate into granulocyte-monocyte progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP). This hypothesis of the hematopoietic hierarchy assumes that the lymphoid system is completely separated from the myeloid system (Figure 1B). However, recent evidence suggests that this separation may not be as absolute as thought earlier, since under some special conditions, early lymphoid cells retain their ability to give rise to myeloid lineage cells. This has been observed both for B lymphoid cells (Schaniel et al., 2002) as well as T lymphoid cells (Bell and Bhandoola, 2008; Wada et al., 2008). Such early lineage infidelity may have clinical significance, since it has been shown that early B cell precursors can give rise to myeloid leukaemia in CALM-AF10 transformed marrow cells (Deshpande et al., 2006). Despite the uncertainties in early lineage diversification, transcriptional regulation of HSC self-renewal and lineage specification is slowly being uncovered. The previously mentioned transcription factors *Runx1* and *Scf1* are both important to maintain the number of HSC, whereas other factors, like *Sfpi1* (the gene encoding transcription factor Pu.1) and *Gata1* may be involved in lineage specification (Laslo et al., 2008; Rosenbauer and Tenen, 2007). One thing that is clear is that HSC reside in the bone marrow as quiescent cells and lineage specification largely takes place within this tissue as well.

Marker	Alternative name	Expressed by	Comments	References
Cd3		Most T cells	Used in lineage cocktail	(Havran et al., 1987)
Cd4		Subsets of T cells		(Spangrude et al., 1988)
Cd5	Ly-1	Most T cells	Used in lineage cocktail	(Ledbetter et al., 1980)
Cd8		Subsets of T cells		(Spangrude et al., 1988)
Cd11b	Mac1	Mostly myeloid cells but also some lymphoid	Used in lineage cocktail	(Spangrude et al., 1988)
Cd16/32	FcgrII/III		Used to distinguish different myeloid progenitor sub-populations	(Akashi et al., 2000)
Cd34	Mucosialin	Endothelial cells, some hematopoietic progenitor cells	HSCs are negative or low in expression	(Osawa et al., 1996)
Cd45R	B220	Mostly B cells	Used in lineage cocktail	(Coffman and Weissman, 1981)
Cd48	Slamf2	Many hematopoietic cells, multi - lineage marker	HSCs are negative or low in expression	(Kiel et al., 2005)
Cd71	Transferrin receptor	Erythroid cell sub population separation, dividing cells	Used for erythroid cell sub populations	(Lesley et al., 1984)
Cd90	Thy1.1	Hematopoietic stem cells and thymocytes	HSC are "low" in expression	(Papathanasiou et al., 2009; Spangrude et al., 1988)
Cd117	Kit tyrosine kinase receptor	Hematopoietic stem/progenitor cells and mast cells	HSCs are positive	(Ikuta and Weissman, 1992)
Cd127	Il7r	Variations of B and T cells	HSCs are negative, used to sort out CLPs from Lin- cells	(Akashi et al., 2000)
Cd135	Fik2/Flt3 tyrosine kinase receptor	Hematopoietic progenitors lacking erythroid and megakaryocytic potential	HSCs are negative	(Adolfsson et al., 2005)
Cd150	Slamf1	B and T cells	Up regulated when cells are activated. HSC are positive	(Kiel et al., 2005) (Papathanasiou et al., 2009)
Cd161	NK1.1, Klr1c	Natural killer cell lineage		(Hackett et al., 1986)
Cd201	Epcr, Procr	Endothelial cells and early hematopoietic cells	HSC are positive	(Balazs et al., 2006)
Gr1	Ly-6G	Granulocytes and monocytes	Used in lineage cocktail	(Spangrude et al., 1988)
Ter119	Ly76	Erythroid cells	Used in lineage cocktail	(Kina et al., 2000)
Sca1	Ly6A/E	Hematopoietic stem/progenitor cells and some myeloid and lymphoid cells	HSCs are positive	(Spangrude et al., 1988)

Table 1. List of hematopoietic surface markers. Antibodies used to distinguish different subsets of hematopoietic cells.

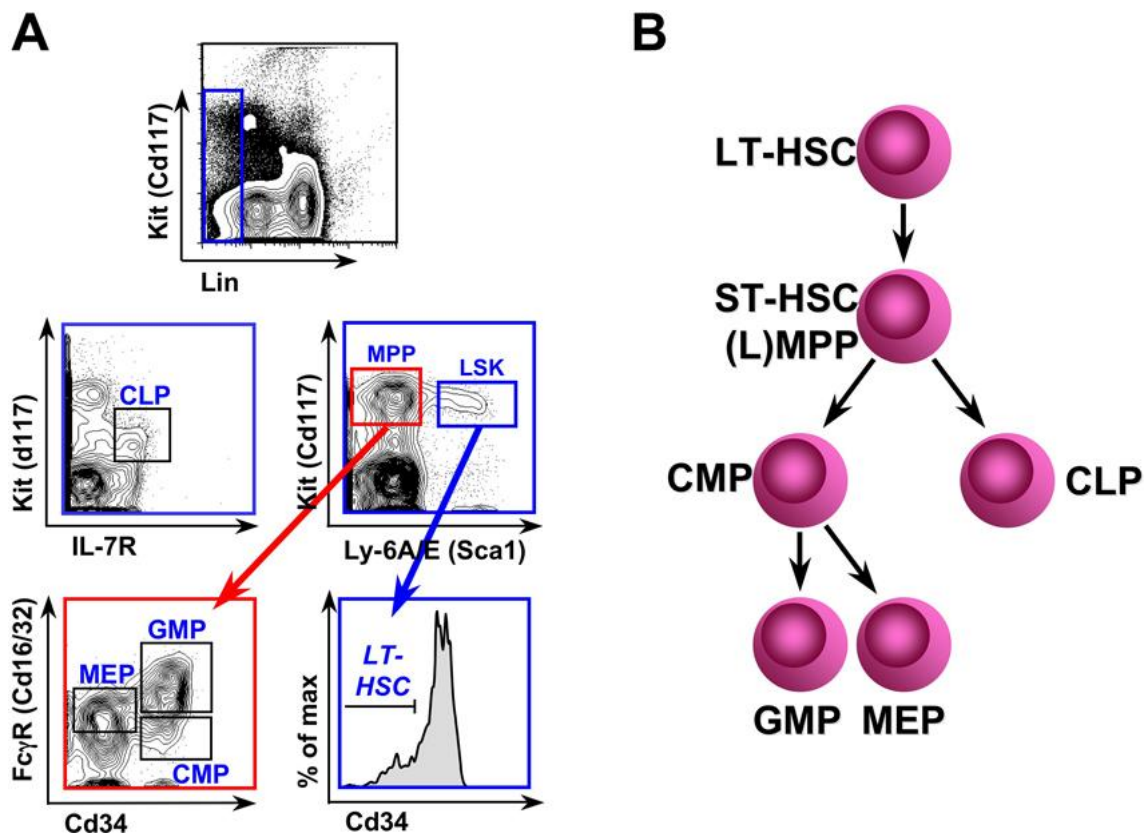


Figure 1. The hematopoietic hierarchy in the adult mouse. The figure shows the different dot plots obtained by phenotypical analysis of whole bone marrow cells using flowcytometry. In A, the breakdown of different subpopulations is shown as described in detail in (Renstrom et al., 2009). In B, the most likely lineage relationships between the cells in A are shown in a small diagram. Abbreviations: HSC: hematopoietic stem cell (LT-: long-term; ST-: short-term). MPP (multipotent progenitor, LMPP: lympho-myeloid potential progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor, GMP: granulocyte-monocyte progenitor, and MEP: megakaryocyte-erythrocyte progenitor.

1.3 The hematopoietic stem cell niche

In adults, normal hematopoiesis takes place in the bone marrow. Schofield was the first to formulate that “the stem cell is seen in association with other cells which determine its behaviour” (Schofield, 1978). This notion, that the stem cells fate is determined by surrounding cell structures, is nowadays referred to as the stem cell microenvironment, or the stem cell niche (Fuchs et al., 2004; Garrett and Emerson,

2009; Morrison and Spradling, 2008; Nilsson and Simmons, 2004; Ohlstein et al., 2004; Spradling et al., 2001). In other organisms, such as *Drosophila* fruit flies (Nystul and Spradling, 2007), the existence of germ cell niches have been well-established. In these germ cell niches, stem cells are propagated by asymmetric cell divisions, where a dividing stem cell gives rise to a niche-attached stem cell and a progenitor cell (Yamashita et al., 2005). The attractiveness of the idea that in the adult hematopoietic system, similar niches exist where stem cells self-renew has spurred many investigators to find the exact location of the niche and define its cellular components.

What is now known is that the most immature HSCs reside most likely near the endosteal region of trabecular bone (Calvi et al., 2003; Zhang et al., 2003). However, the search for the exact location of the niche and the cell types involved is still not resolved completely. The endosteal region is actively recruiting HSC, probably by secreting factors like the chemokine Cxcl12. Within hours after intravenous transplantation, HSC localize within microns of the endosteal surface, though only a small percentage of cells will localize directly adjacent to the osteoblasts (Lo Celso et al., 2009; Xie et al., 2009). Two types of niches have been described: the endosteal niche, where the HSC remain close to osteoblasts of trabecular bone and a perivascular niche, where the stem cells are closer to vascular endothelium in marrow sinuses (Kiel and Morrison, 2008). Whether these are factually different niches, or whether the two niches are one and the same, remains subject to speculation (Garrett and Emerson, 2009).

Another area of intense investigation revolves about the question how the niche directs self-renewal and differentiation of HSC. Most studies have focused on integral parts of the niche, the osteoblasts and bone marrow stroma cells. It has already been shown that these cells can secrete factors such as G-CSF, GM-CSF, Kitl (stem cell factor, SCF), Il-6 and Sdf1 which influences hematopoietic cell fate both in vitro and in vivo (Jung et al., 2008; Taichman and Emerson, 1994; Taichman et al., 1997; Zhu et al., 2007). Using the knowledge of which secreted factors are generated by stromal cells, have so far not led to stroma-free culture conditions of HSC expansion. The reason for that might be that stromal activation after environmental insult may be required for short-term HSC expansion. However, the factors governing a return to G0/G1, a prerequisite for long-term maintenance of HSC, are unknown at present. Also, after insult, HSC may be released from the “maintenance niche” and move closer to the “expansion niche” where proliferation and differentiation takes place (Lo

Celso et al., 2009; Xie et al., 2009). The idea that HSC are, in fact, subject to the influence of different cellular environments, each with different functional relevance, has received impetus by observations that activated (leukemic) cells, can displace quiescent HSC, thereby dysregulating their proliferative behaviour (Colmone et al., 2008).

Thus, in vivo imaging suggests that HSC may occupy several different niches in the bone marrow, depending on the activity of the HSC or its direct progeny at a given time. The picture of the niche, as first formulated by Schofield, has now become focused on the endosteal surface of trabecular bone, where both endosteal and vascular niches may exist.

1.3.2 Microenvironment-dependent signals which regulate hematopoiesis

It has been shown that most HSC remain quiescence (i.e. in G0/G1 phase of the cell cycle) at any given time (Fleming et al., 1993), but will rapidly respond to hematopoietic stress induced by disturbances of the hematopoietic system. Homeostasis thus depends on a tightly regulated balance between self-renewal, proliferation, and differentiation of HSC and its daughter cells. In order to keep this homeostasis, mechanisms have to be in place to insure that HSC return to quiescence with the help of several signalling molecules from a number of different biological pathways. Several pathways have, in fact, been studied for their role in niche-HSC in these activating and de-activating regulatory mechanisms, including the Tie2/Ang1-N-cadherin/Myc axis, Bmp/Tgf signalling, Sdf1/Cxcr4 signalling, hedgehog and Notch signalling, as well as Wingless (Wnt) signalling.

Examples of gene products involved in niche-HSC interaction are Tie2 and its ligand Angpt1, which are both required to maintain quiescence of HSC at the endosteal interface (Arai et al., 2004). One target of Tie2 signalling, N-cadherin, has also been implicated in niche-HSC interactions and seemed to be involved in possible asymmetric cell divisions (Wilson et al., 2004). The central role of N-cadherin could, however, not be confirmed in mice deficient in N-cadherin, since bone marrow cellularity, progenitor activity, HSC function or numbers are unchanged by the absence of N-cadherin (Kiel et al., 2009). A second pathway involved in stem cell regulation is the Bmp-Tgf pathway. It has been long since known that Bmp4 is not only involved in the hematopoietic specification of embryonic stem cells (Chadwick et al., 2003). But, Bmp4 has also been implicated in stromal cell regulation of embryonic

definitive HSC (Durand et al., 2007) as well as adult HSC in vivo (Goldman et al., 2009). The responsible Bmp receptor has not yet been identified, but a good candidate is *Bmpr1a* which has now been found to be strongly expressed by osteoblasts, and its deficiency decreases the number of transplantable HSC (Zhang et al., 2003). A third pathway of niche-HSC regulation may include stromal derived factor 1 (*Sdf1*, *Cxcl12*) and its receptor *Cxcr4* (Kopp et al., 2005). *Sdf1* is produced by stromal cells in the niche and *Cxcr4* is strongly expressed on the earliest HSC. *Sdf1* is a chemokine which is thought to be involved in regulating the number of HSC in the circulation (Lyman et al., 1993; Yin and Li, 2006), primarily by regulating the attachment of HSC to the niche through Mmp9- and Kit-dependent proteolytic release (Heissig et al., 2002). By releasing *Cxcr4* expressing cells, not only is their trafficking disturbed, but the released HSC also change their cell cycling activity, suggesting that *Cxcr4* may be involved in maintaining HSC quiescence (Nie et al., 2008). A fourth pathway is the hedgehog (*Hh*) signalling pathway, which is governed by the signalling intermediate smoothed (*Smo*). Like in the case of N-Cadherin, however, hedgehog signalling does not seem to be involved in the maintenance of HSC in *Smo*-deficient mice (Gao et al., 2009; Hofmann et al., 2009). Two pathways we will discuss in more detail: the Notch and Wnt-pathways. Current evidence suggests, that these two pathways find themselves very often collaborating with the other mechanisms described previously as well as with each other. The Notch and Wnt pathways may therefore form a possible interface in niche-mediated regulation of early hematopoiesis.

1.3.3 Notch signalling

In experiments designed to find out which role Notch signalling plays in hematopoiesis, it was shown that over expression of the active intracellular Notch1 domain increased self-renewal of LSK cells (Varnum-Finney et al., 2000). Furthermore, the interaction between the Notch ligand Jagged1 and Notch1 impairs differentiation both in vitro (Li et al., 1998; Stier et al., 2002), and in vivo (Stier et al., 2002). Similarly, ectopic expression of *Dll4* in stromal cells will increase human progenitor cell maintenance in vitro (Dando et al., 2005) It was later also shown that *Notch1* is expressed by HSC. Interestingly, Notch signalling intermediates, like *Notch1*, *Notch3*, and its ligands *Jag1* and *Dll1*, are expressed by osteoblasts (Calvi et al., 2003; Nobta et al., 2005), suggesting Notch-mediated HSC-niche crosstalk. Experiments with floxed *Jag1* or *Notch1* mice or deficiency of Notch signalling

through over expression of a dominant-negative variant of the central Notch intermediate mastermind-like 1 (*Maml1*) indicate, however, that signalling through this receptor-ligand pair is not necessary for maintenance of HSC and hematopoiesis under steady-state conditions (Maillard et al., 2008; Mancini et al., 2005). Thus, the exact role of Notch1 signalling, or the role of other Notch receptors (particularly *Notch3*) in microenvironmental regulation of HSC remains unclear. What is clear, is that Notch signalling cooperates with other signalling pathways during hematopoietic regeneration (Kim et al., 2009). In particular, crosstalk between Notch and Wnt-signalling was shown to occur. Interestingly, though most studies have concentrated on intrinsic HSC signalling, recent evidence shows that Wnt and Notch crosstalk may occur in an extrinsic manner (Yamane et al., 2001), where stroma cells containing activated β -catenin (*Ctnnb1*) up regulated Notch signalling in hematopoietic cells during stress conditions such as myeloablation. Thus, it seems likely that Notch signalling is important in niche-HSC interactions, particularly during hematopoietic regeneration.

1.3.4 Wnt signalling

Members of the Wingless (Wnt) family of lipid-modified proteins have been investigated best in hematopoiesis (Hausler et al., 2004; Kim et al., 2009). The Frizzled (Fzd) receptors act as Wnt receptors which activate downstream signalling in the *Ctnnb1*-dependent canonical and non-canonical pathways. Canonical Fzd receptors associate with the Lrp5/6 co-receptors, and propagated signals through catenins, to activate Tcf/Lef transcription complexes. This transcriptional complex targets transcription of specific genes, including *c-myc*, *Spp1*, *Socs2*, *P2ry14* and *Ccnd1* (Nygren et al., 2007). The level of *Ctnnb1* is regulated through the proteome. In non-canonical Wnt signalling Fzds and non-related Ryk or Ror receptor tyrosine kinases activate calmodulin/ Ca^{2+} - or Rho-dependent responses. These pathways regulate a different set of Wnt targets, such as *Pparg* and *Pcdh8* (Takada et al., 2007). Also, there is interaction between non-canonical and canonical pathways, as non-canonical Ca^{2+} -dependent signals inhibits catenin stability through Camk2-mediated activation of Nemo-like kinase (*Nlk*) and subsequent phosphorylation of *Ctnnb1*, which is then degraded (Ishitani et al., 2003).

Several components of the Wnt signalling machinery have been shown to play a role in HSC self-renewal. Both canonical as well as non-canonical pathways seem to be involved, since the canonical ligand *Wnt3a* intrinsically promotes self-renewal (Luis et

al., 2009). On the other hand, the non-canonical ligand *Wnt5a* has been shown to extrinsically promote self-renewal, but, paradoxically enough, by inhibiting canonical signalling (Murdoch et al., 2003; Nemeth et al., 2007). The mechanistic basis for the balance between canonical or non-canonical pathways is not fully understood. It is likely that the numerous Wnt-signalling inhibitors (Dickkopf homolog (*Dkk*), Wnt inhibitory factor (*Wif*) or Secreted frizzled related protein (*Sfrp*), or other Wnt antagonists, such as Kremen, *Ctgf*, *Cyr61*, *Wise* and *Sost*, are modulating Wnt signalling. Interestingly, some of these also directly stimulate certain Fzds independent of Wnt factors. For example, *Sfrp1* directly activates *Fzd2* (Rodriguez et al., 2005) , as well as *Fzd4*, and *Fzd7* (Dufourcq et al., 2008) but can also interact with *Wnt5a* (Matsuyama et al., 2009).

This balance and feedback mechanisms between canonical and non-canonical Wnt signalling, suggests that *Ctnnb1* is the primary regulatory target of Wnt signalling. However, over expression or stabilization of *Ctnnb1* results in expansion of the HSC pool, but, at the same time, to loss of myelopoiesis due to a differentiation block (Kirstetter et al., 2006; Scheller et al., 2006), suggesting that *Ctnnb1* promotes self-renewal and/or inhibits differentiation. Surprisingly, conditional deletion of *Ctnnb1* or both *Ctnnb1* and plakoglobin (β -catenin, *Jup*) does not affect the repopulating ability of HSC (Cobas et al., 2004; Jeannet et al., 2008; Koch et al., 2008). Thus, it appears that *Ctnnb1* does not have intrinsic effects on HSC maintenance and engraftment.

The role of catenin suggests different levels of hematopoietic regulation. Although the above indicates that both β - and γ -catenin are dispensible for normal hematopoiesis, this appears not to be true for leukemic stem cells. *Ctnnb1* and other Wnt intermediates are frequently over expressed in leukemic patient samples (Muller-Tidow et al., 2004). The importance of *Ctnnb1* in leukemia is also highlighted by the observations that missplice mutations of its upstream kinase *Gsk3b* (Abrahamsson et al., 2009), or over expression of its downstream mediator *Lef1* (Petropoulos et al., 2008) cause myeloproliferation. In addition, Bcr-Abl1 leukemia requires *Ctnnb1* expression (Zhao et al., 2007), probably by conferring self-renewal ability to the mature GMP population (Minami et al., 2008). Taken together, current reports suggests that *Ctnnb1*, and perhaps other catenins, may play different roles in normal as compared to leukemic HSC, where *Ctnnb1* is dispensible or redundant for normal HSC but required for leukemic HSC behaviour.

The above findings all deal with the possibility of Wnt signals within the stem cells (intrinsic signals). However, there is also data showing that Wnt signalling is important in bone formation and enlarging endosteal surfaces (reviewed by (Yin and Li, 2006)). Several lines of evidence suggests that Wnt signalling in endosteal stromal cells may affect HSC maintenance, not by intrinsic signals, but by signals from the stromal cells (extrinsic signalling). For instance, increased expression of *Ctnnb1* in marrow stroma improves HSC maintenance and concomitantly increases HSC engraftment (Kim et al., 2009; Nemeth et al., 2009). Although the precise mechanism of this effect has not been identified, increased Notch signalling was demonstrated in the HSC cocultured on *Ctnnb1*-overexpressing stromal cells (Kim et al., 2009). Moreover, further evidence comes from mice over expressing the canonical inhibitor *Dkk1* in stromal cells of the niche, the HSC pool is gradually lost (Fleming et al., 2008). Also, stroma cells from mice deficient in the non-canonical Wnt mediator *Nlk* were shown to be defective in maintaining hematopoietic progenitors due to extrinsic effects (Kortenjann et al., 2001).

Taken together, Wnt signalling may not be intrinsically involved in the maintenance of normal HSC during hemostasis or self-renewal. However, there is data suggesting that changes of Wnt signalling in endosteal stromal cells affect HSC maintenance through extrinsic mechanisms. Future studies should more clearly define the spider web of interactions that operate in stromal cells and HSC and which, together, are involved in HSC regulation.

1.4 Dysregulation of the niche may cause hematopoietic abnormalities

Myeloproliferative disease has been shown to be primarily caused by the faulty expression of mutated growth factor receptors or the formation of fusion oncogenes, which cause an intrinsic gain of self-renewal and proliferative capacity. Yet, there are also myeloproliferative conditions where no apparent changes in HSC can be found, such as in myelodysplastic syndromes. Indeed, only recently has it been recognized that changes in the niche may, in fact, cause myeloproliferation. For instance, loss of the NFkB signalling intermediate *Ikba* causes a stroma-dependent increase in myeloproliferation, which is partly due to increased stromal *Jag1* expression and *Notch1* signalling in myelocytes (Rupec et al., 2005). Another study which points to the Notch pathway are conditional knockouts for the Notch pathway mediator mindbomb homolog 1 (*Mib1*). In these animals, though, suppression of Notch

signalling in the microenvironment causes a change in extrinsic cues culminating in myeloproliferation (Kim et al., 2008), suggesting that up regulating Notch in either HSC or stromal cells may have opposite effects.

Unanticipated was also that deficiency of the cell cycle regulators *Rb* (Walkley et al., 2007b) and *Rarg* (Walkley et al., 2007a) causes a niche-dependent myeloproliferation. In these models, *Rb* impairs lodgement and homing of HSC, whereas, *Rarg* disturbs Tnf-mediated signalling.

Lineage decisions are also suspected to be directed by microenvironmental cues. A recent study elegantly showed that certain cytokines direct CMP towards either granulocytes or monocytes (Rieger et al., 2009). It has been shown that mice deficient in the telomere component *Terc* show increased stromal production of G-CSF and an increased proportion of Gr1-expressing myelocytes (Ju et al., 2007), confirming that the niche directs cell fate decisions in the myeloid lineage. It is of interest to note that in disease, the niche may similarly direct the phenotype of myeloproliferative disease. This has been shown to occur in MLL-AF9 expressing cells which form either mixed lymphoid-myeloid leukaemia, or myeloid leukemia, depending on the growth factors expressed by the microenvironment (Wei et al., 2008).

Taken together, these studies show that disruption of the expression of certain genes in the niche may not only cause myeloproliferative disease, but may also direct the lineage fate of HSC development, thereby determining the phenotype of the disease formed from the leukemic stem cell.

1.5 Summary and conclusions

The regulation of hematopoietic stem cell activity through its surroundings, or niche, is a concept, first proposed in the 70's by Schofield (Schofield, 1978). Investigations into how the components of the niche influence HSC are unravelling the mechanisms involved. The identification of single HSC by their surface phenotype, has facilitated specific studies in lineage relationships and self-renewal of single HSC. It has become clear that the HSC can be found near the endosteal region of the bone possibly in the vicinity of blood vessels. Whether there is a need for direct cell to cell contact between the HSC and its surrounding niche cells, possibly through integrin alpha 4 and alpha 9 mediated interactions (Grassinger et al., 2009; Oostendorp and

Dormer, 1997) has still not been resolved decisively. In order to investigate the intricate interplay between niche and HSC, many researchers use cell lines, capable of maintaining HSC activity in vitro, which mimics the niche and serve as homogenous models which can be taken apart in more detail (Muller-Sieburg and Deryugina, 1995; Oostendorp et al., 2002; Wineman et al., 1996). These studies are uncovering possible mechanisms by which the niche regulates self-renewal, quiescence and proliferation of HSC. Furthermore, these studies now allow a modified hypothesis of the cause of myeloproliferation. Whereas it has long since been recognized that intrinsic changes of HSC may cause myeloproliferation (Figure 2A), it has also become clear that changes induced in the stromal compartment of the niche may also lead to myeloproliferative disease (Figure 2B). All of this highlights the role of the niche in maintaining a steady state hematopoiesis which is essential for our continued well being.

1.6 Aim of thesis

The aim of this thesis is to investigate how the microenvironment influences hematopoiesis through extrinsic means. As reviewed above, though more and more is known about intrinsic mechanisms of HSC regulation, little is known about how such intrinsic signals are directed or modulated from the outside, through extrinsic processes. To identify possible extrinsic modulators, we have at our disposal stromal cell lines which maintain HSC in culture, and as such are capable of mimicking the microenvironment in vitro. In this thesis, we will attempt to identify these extrinsic modulators using yeast two-hybrid screening and micro-array-based gene expression studies. When suitable candidates for such interactions are found, they will be further tested to pin down their exact role in the complex hematopoietic machinery, primarily by using mouse models, but also, in collaborative studies, by using human ES cells. In these studies, we will focus on the questions: are the identified factors influencing hematopoiesis in vivo? If so, which part of the hematopoietic hierarchy do they affect, mature or immature cells? Furthermore, we are very interested through which signalling pathway hematopoiesis is primarily affected.

Answering such questions will give us knowledge about extrinsic factors that can in the future be used as the basis for new treatments for different hematopoietic ailments.

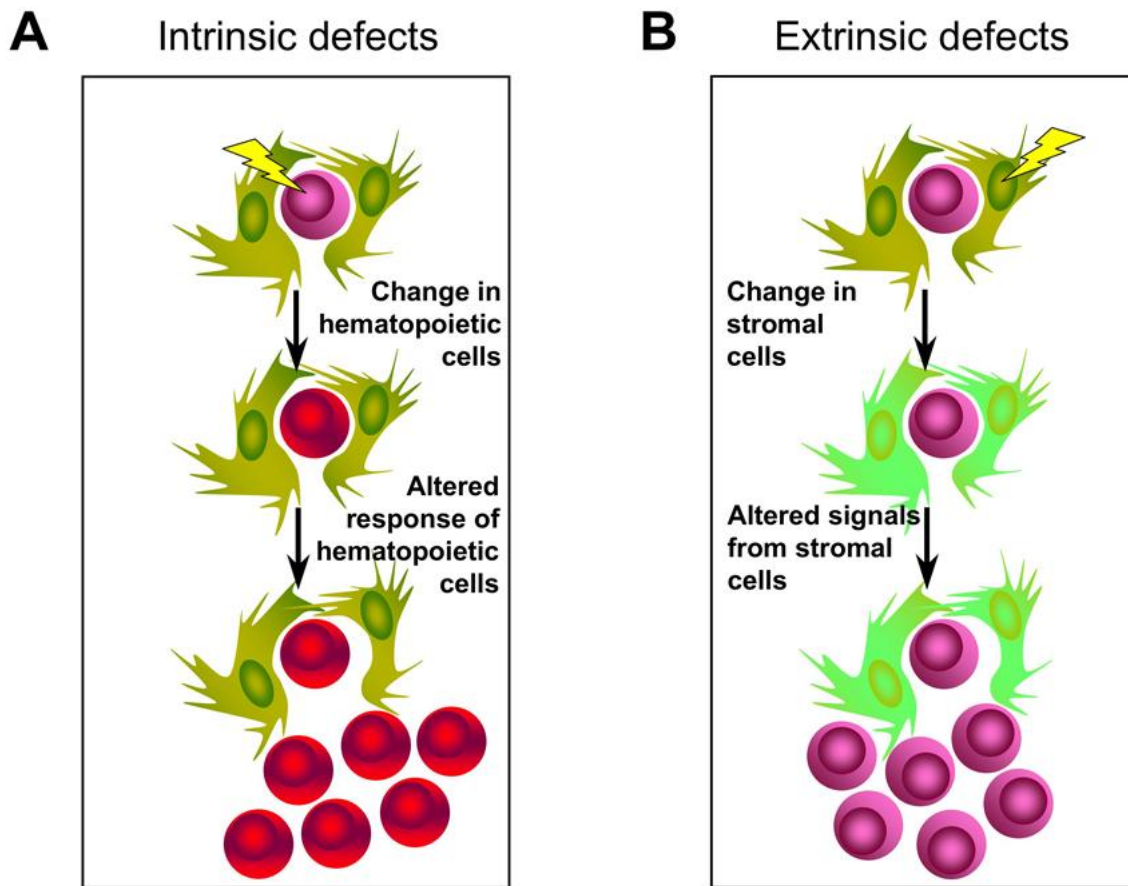


Figure 2. Different theories of how hematopoietic stem cells could be deregulated after intrinsic (A) or extrinsic damage (B). Damaging factors could for instance be: irradiation, cytotoxic agents, or environmental pollutants (like benzene). In A, these damaging conditions cause intrinsic damage, for instance by inducing DNA breaks or other damage, which results in an intrinsically changed hematopoietic cell. This changed cell does not respond correctly to quiescence-inducing signals from the niche. As a result, the changed HSC will continuously proliferate, which may lead to myeloproliferative disease. In B, the damaging conditions affect the stromal cells. In this scenario the stromal cells are changed and cannot give the HSC the correct signals to keep them quiescent. As in scenario A, the HSC will start to proliferate, which could ultimately result in myeloproliferative disease. Thus, two fundamentally different scenarios lead to the same end-result.

Conflict of interest

None

1.7 Acknowledgements

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Chapter 2: Previous work

2.1 Studies prior to my arrival

Prior to my arrival the lab of Dr. Robert Oostendorp has published a series of publications highlighting the HSC maintaining capacity of two cell lines created by Dr. Oostendorp, the UG26-1B6 and EL08-1D2. These cell lines were isolated out of an initial 100 stroma cell clones originating from different sub regions of the tsA58 transgenic murine AGM; the urogenital ridges (UG), aorta/mesenchyme (AM), embryonic liver (EL) and gastrointestinal tract (GI). In some of the first experiments, involving human cord blood derived CD34+ cells, showed that some cell lines were capable of expanding progenitor cells by up to 800-fold over the course of 6 weeks of culture and could maintain the production of early human progenitors over a 12 week period (Oostendorp et al., 2002).

Further studies with murine stem cells showed that the UG26-1B6 cell line could successfully maintain murine as well as human progenitor activity over a period of at least 4 weeks, suggesting a similar effect by factors produced by both human and mouse HSC supporting stroma cells. What was also discovered was the fact that actual contact between that stroma (UG26 and EL08) and the hematopoietic cells was not actually required for the maintenance of hematopoietic progenitor activity (Oostendorp et al., 2005), Figure 3, this was in contrast to other cell lines capable of maintaining hematopoiesis in culture; the AGM-S3, DAS104-4, and AFT024 cell

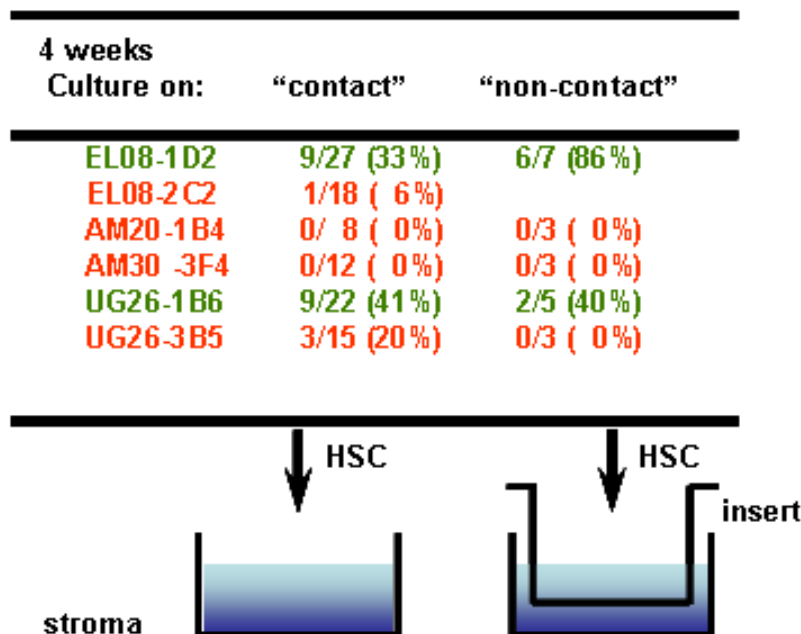


Figure 3. Non-contact cultures. Novel murine stromal cell lines capable of maintaining progenitor activity in a non-contact manner in vitro. Adapted from (Oostendorp et al., 2005)

lines. This is of great importance, since it is based on this notion that soluble factors greatly influence the fate of hematopoietic stem cells, and from a clinical point of view the manipulation of such a system could possibly be achieved with the appropriate pharmaceutical compounds as means to treat hematopoietic ailments. However, the actual proteins involved in this were still unknown and in an attempt to find out possible candidate genes it was decided that the mRNA from these supportive and non-supportive cell lines should be compared using gene expression arrays, in this particular case some 2400 gene “ClonTech Atlas Mouse 1.2 and 1.2II” arrays. The initial analysis found clear similarities between the different cell lines, be it supportive or non supportive, such as the common expression of *BMP-1*, lectin galactose binding soluble proteins 1 (*Lgals1*) and *Lgals3*, *Ccl7*, *Csf1*, *Sdf3*, *Tgfr*, *Vegfa* and *Vegfd*. But there were also significant differences, for instance, both HSC-supportive clones over express pleiotrophin (*Ptn*), insulin-like growth factor binding protein (*Igfbp3*) and *Igfbp4*, thrombospondin 2 (*Thbs2*) and Secreted frizzled related protein 2 (*Sfrp2*), all of which, along with *Gpx3* which was down regulated, were later confirmed with real time PCR and ELISA, Figure 4. In short, available at my arrival were two cell lines, both proven supporters of both murine and human hematopoiesis, and a database with expression data of genes involved with the maintenance of hematopoiesis by these cells. And of course the “know how” concerning the handling of these stroma cells and murine hematopoietic cells. This enabled me to quickly familiarize myself with the methods used and where to start looking for new clues in this great puzzle called hematopoiesis.

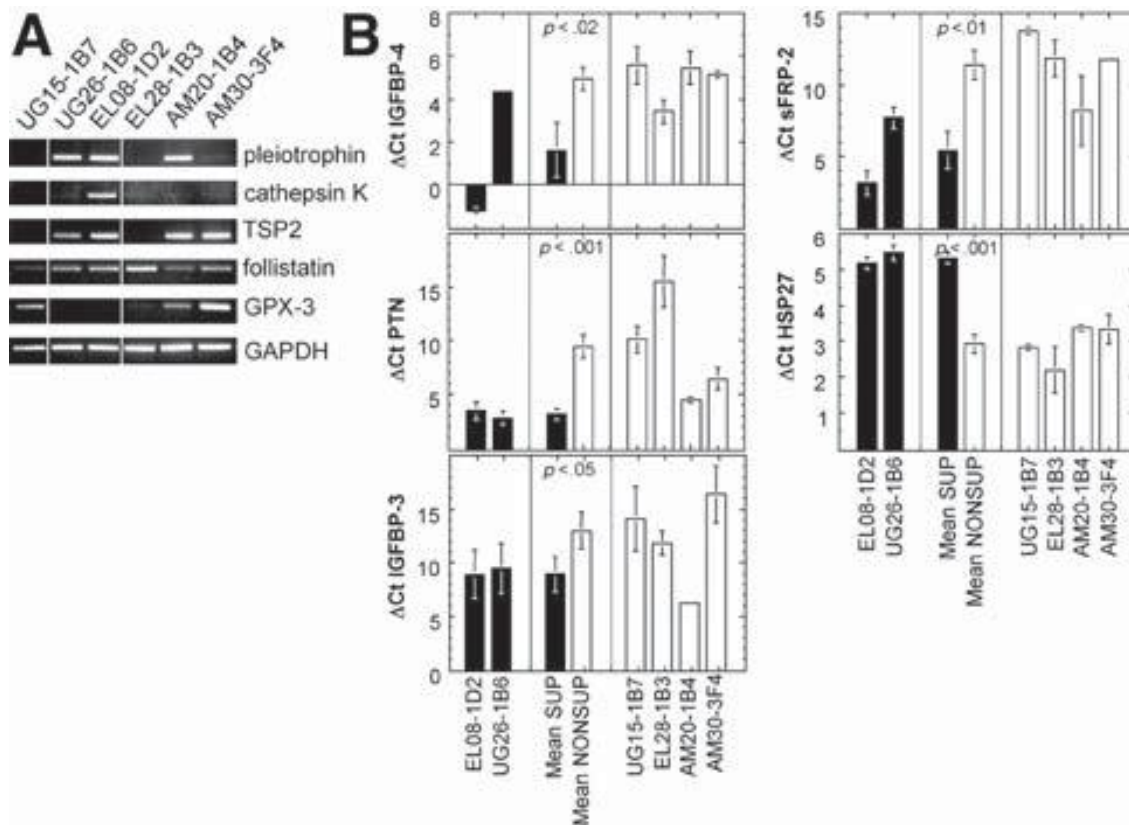


Figure 4. Confirmation of gene expressions. RT-PCR and real time PCR analysis of gene expression in the ClonTech Atlas Mouse 1.2 and 1.2II arrays, from Oostendorp et al., 2005b.

2.2 References

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Chapter 3: Identifying new regulators of hematopoiesis

3.1 Yeast 2 hybrid and Mouse Atlas arrays

The starting point for these experiments was data obtained in gene expression arrays (ClonTech Atlas Mouse 1.2 and 1.2II) which were based on mRNA harvested from two cell lines shown to support hematopoiesis in a non-contact manner and four cell lines that could not support non-contact mediated hematopoiesis.

In order to validate findings from the first array, made by members of the Oostendorp group prior to my arrival in 2005, it was decided that a yeast 2 hybrid screen should be performed on two of the most promising genes found in these arrays. To find out which genes were in fact the most promising the raw data obtained from the arrays, in the form of Excel sheets, was used to compare the genes with each other. In the Excel data there were values for the relative signal intensity for each gene, these could in turn be used to compare the expression values for gene X in the supportive stroma cell lines with the ones from the non supportive cell lines. This was made by linking the Excel sheet to a program called File maker Pro 7 (File maker, Inc, Santa Clara, USA) in order to visualize the calculations made concerning the significance of the expression data, which used the Students T-test as a way of showing significance. Using this method; two genes were considered to be more promising than the others, these were: Secreted frizzled-related protein 2 (*Sfrp2*) which is a 33kDa, Wnt-antagonist shown to be involved in somitogenesis and anterior/posterior pattern formation; and Pleiotrophin (*Ptn*), a 19kDa heparin binding protein involved in many processes such as bone mineralization, learning and cell proliferation. The reason why these genes attracted our attention was that both of them were highly over expressed (more than 3 fold) and that both were soluble secreted proteins. The fact that they are soluble was one of the core issues in this project since it would require a soluble factor to be produced by the supportive cell lines which in turn would affect the hematopoietic stem cells to maintain them in a non-contact culture. In order to further investigate the way these two proteins could influence hematopoiesis it was decided to try and use these genes in a Yeast 2 hybrid screen, which is used to find previously unknown interaction partners of the genes analyzed. The conclusion was that the MATCHMAKER GAL4 two-Hybrid System 3 (Clontech, Mountain View, USA) kit should be used for these experiments. The theory behind yeast 2 hybrid is that the gene of interest is cloned into a vector which fuses a Gal4 DNA-binding domain (DB) to the protein and that this fusion protein will then interact inside a yeast cell with its interaction partner, which is fused with a Gal4 activation

domain (AD), and these interaction partners will come from a “genetic library” constructed from hematopoietic cell mRNA. The interaction of these proteins in the yeast nucleus will cause the DB and AD to get into close proximity of each other and thus starting the transcription of 4 different reporter genes. Colonies of yeast cells containing the interacting genes can then be grown on culture plates lacking these 4 reporter genes in order to make sure that the interaction is a real one, Figure 5. Colonies found to grow in condition that required the interactions between two constructs were then sequenced in order to reveal the identity of the interacting partner of our target protein.

Experiments using *Sfrp2* and *Ptn* as bait yielded some possible interaction partners; *Vav1* and 2210010N04Rik for *Sfrp2* while *Anapc2*, *Atp5a1*, *Pias3*, *Sgol1* and *Ap1g2* were detected for *Ptn*.

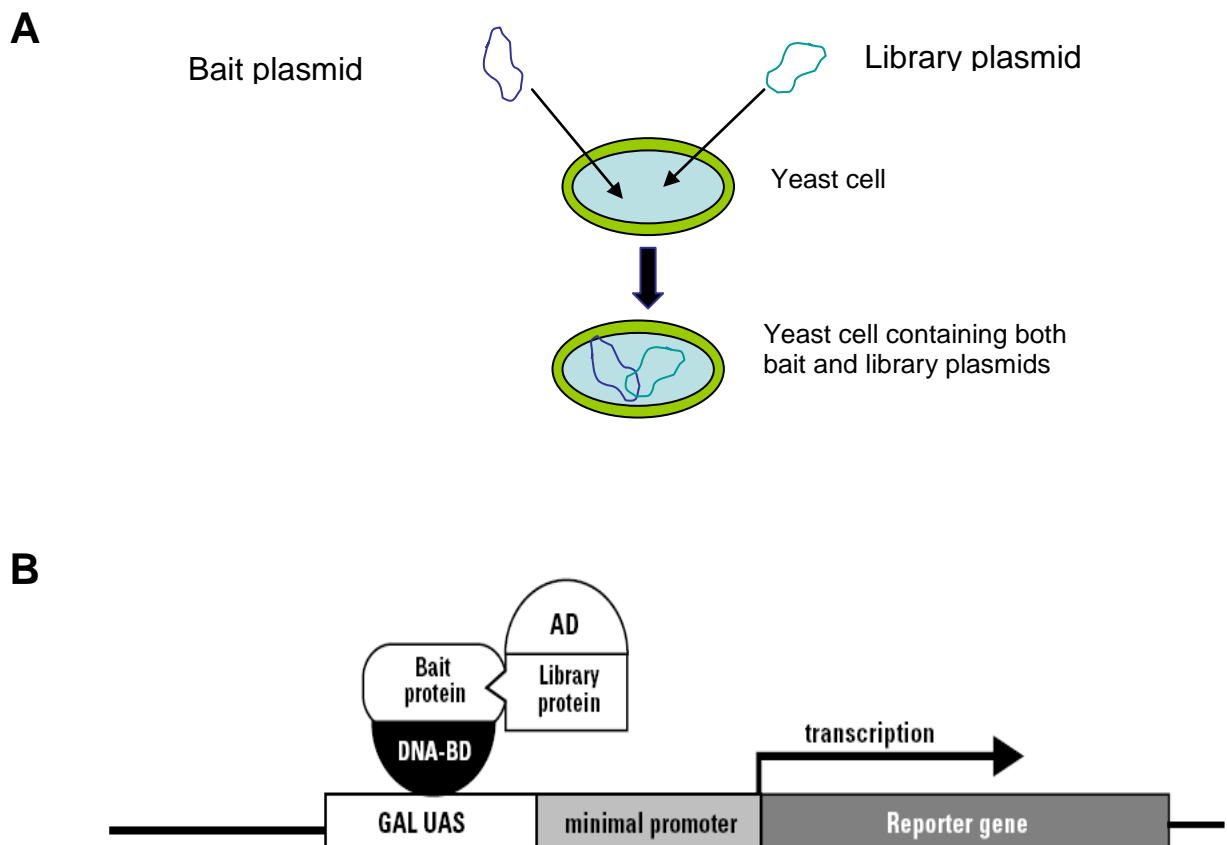


Figure 5. Principle of yeast 2 hybrid. A) The bait and library gene plasmids inserted into the yeast cell. **B)** Protein interactions resulting in the transcription of the reporter gene.

3.2 Old array data results

The data generated by this earlier gene expression array (ClonTech Atlas Mouse 1.2 and 1.2II arrays) was then organized using the File maker Pro7 programme. To find suitable candidates the data was compared between the two supportive cell lines UG26 and EL08 and the other non-supportive ones (EL28, AM20, AM30 and UG15) on the basis of expression levels (normalized), fold changes in expression levels and significance values, for the individual supportive lines and also comparing both of them as “one” in comparison to the others, Table 2.

Gene	Swissprot	Expression	Fold change	P-value	Location
<i>Shox2</i>	P70390	3,28	2,63	0,008	Nucleus
<i>Il1r1</i>	P13504	3,59	2,21	0,002	Membrane
<i>Tgfb3</i>	O88393	4,14	2,25	0,011	Membrane, extracellular space, cytoplasm
<i>Dll1</i>	Q61483	2,59	2,07	0,0002	Membrane
<i>Ccl7</i>	Q03366	4,61	2,72	0,011	Secreted
<i>Ptn</i>	P20935 (new P63089)	4,51	6,14	0,003	Secreted
<i>Fgf9</i>	P54130	2,99	2,75	0,003	Secreted
<i>Fst</i>	P47931	4,15	2,94	0,009	Secreted
<i>Igfbp6</i>	P47880	4,41	2,87	0,016	Secreted
<i>Igfbp3</i>	P47878	5,11	4,57	0,005	Secreted
<i>Igfbp4</i>	P47879	3,96	6,60	0,002	Secreted
<i>Sfrp2</i>	O35297	3,24	3,67	0,011	Secreted
<i>Il6</i>	P08505	2,96	3,10	0,019	Secreted
<i>Thbs2</i>	Q03350	4,07	2,72	0,004	Extracellular region
<i>Fmr1</i>	P35922	4,09	2,15	0,005	Cytoplasm, Nucleus
<i>Ptx3</i>	P48759	7,46	2,89	0,037	Secreted
<i>Aqp1</i>	Q02013	5,37	2,03	0,020	Membrane
<i>Ins1</i>	P01325	5,33	2,49	0,004	Secreted
<i>Cfd</i>	P03953	5,46	2,64	0,009	Secreted
<i>Ctsk</i>	P55097	6,27	3,85	0,0001	Lysosome

Table 2. Some of the over expressed genes found in the first gene expression array (ClonTech Atlas Mouse 1.2 and 1.2II). The criteria were as follows: Expression >2, Fold change >2 and a P-value <0,05. P-value was obtained when combining both the supportive cell lines in the comparison to the other cell lines.

Out of these genes those that were not secreted were excluded, for example *Fmr1* and *Aqp1*, since a protein must be secreted in order to give the results seen in the non-contact cultures. Due to several scientific and practical reasons, such as if there were known hematopoietic effects of the genes or their signalling pathways and the amount of already published data available in 2005, the focus ended up being on two genes: *Ptn* and *Sfrp2*.

3.3 Yeast 2 hybrid results

After initially creating the plasmids used as bait and library constructs, these plasmids were inserted into the AH109 yeast strain. Preliminary results showed great promise with several colonies growing on agar plates deficient in the His, Leu and Trp, Figure 6.

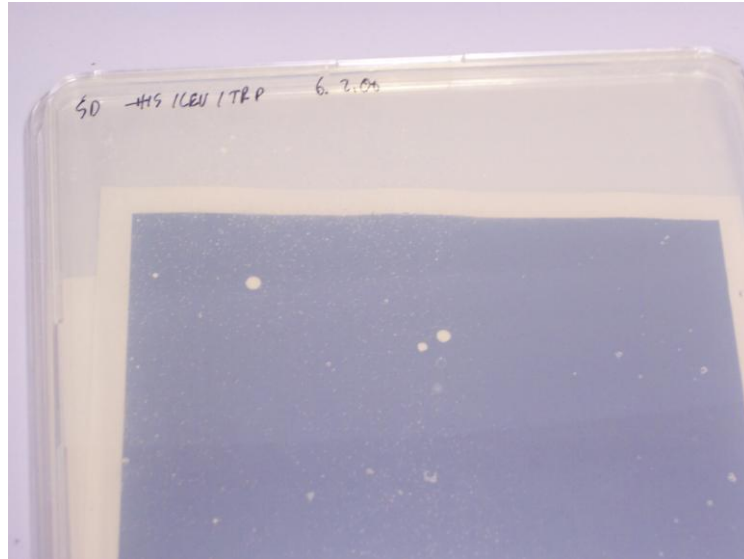


Figure 6. Preliminary results from the Ptn yeast 2 hybrid screen. Yeast colonies can be seen as white, round colonies.

These colonies were then isolated, the plasmids inside extracted and amplified using a bacterial cloning system. These sequences were sent to a sequencing company (GATC Biotech) who sequenced the inserts in both the pGADT7 (GAD) and pGBKT7 (GBK) vectors. The GAD vector would then contain a sequence from the gene which produced the peptide that interacted with the peptide formed by our Ptn or Sfrp2 sequence incorporated into the GBK vector. Both library (GAD) and bait (GBK) vectors were sequenced to ensure that the bait sequence was a perfect match with the gene investigated using the Yeast 2 hybrid system. An example of GAD sequences digested with by the Sfi1 enzyme in order to identify clones of different insert size can be seen in Figure 7. This was easily done by boiling yeast cells picked from a colony in the microwave prior to digestion.

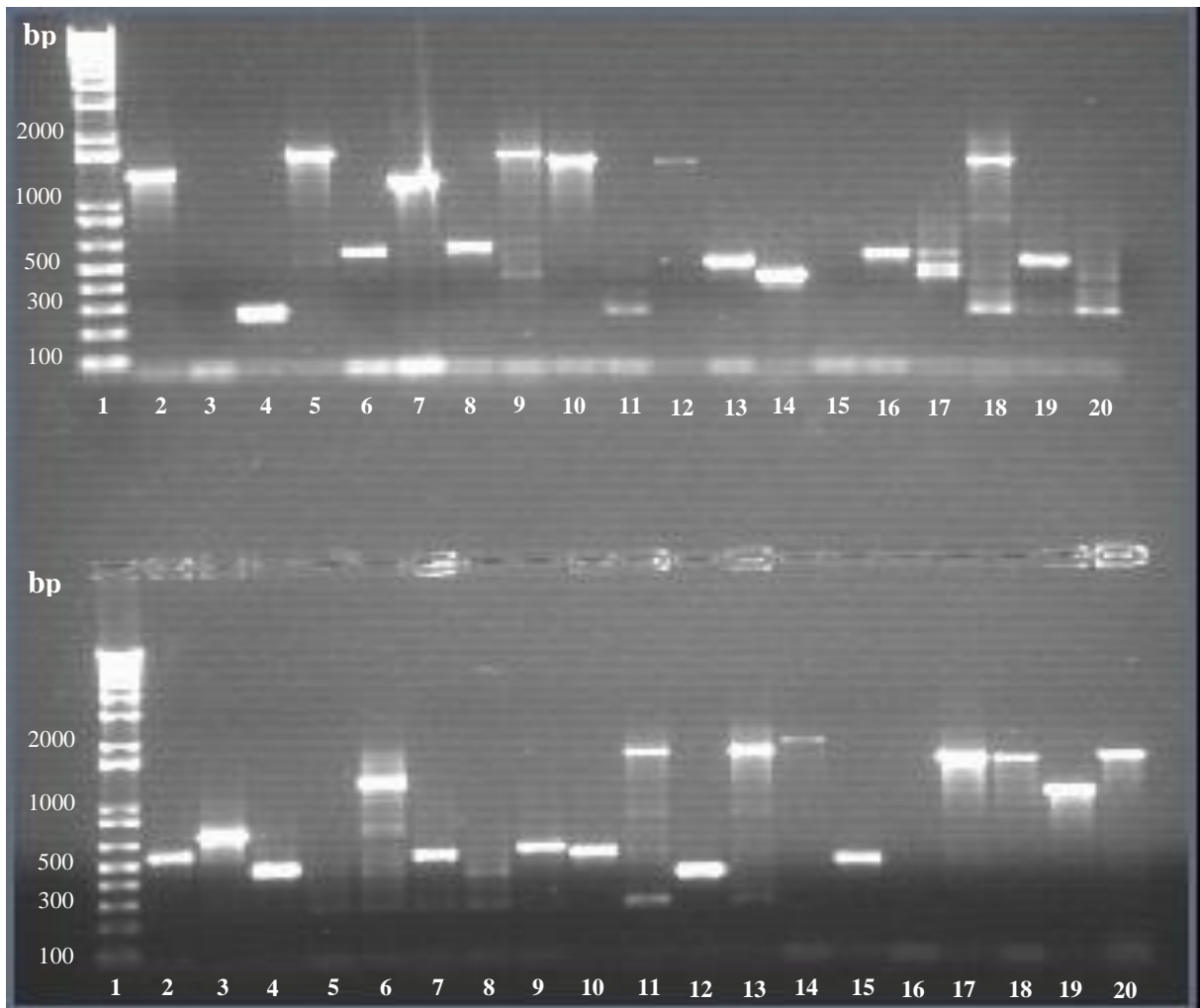


Figure 7. Gel picture of SfiI digest. Different sized inserts, in base pairs (bp), from GAD vectors taken directly from boiled yeast cells, lanes 1-20 upper and lower lanes, digested by SfiI.

The sizes of the inserts were then manually sorted in order to distinguish the different sequences from each other, after which one or more examples of a clone were sent for sequencing at GATC Biotech. When it had been established that the insert of the GAD vector was a gene of interest and not just the result of a faulty culture or insertion, this due to the fact that some colonies grew despite not having any detectable inserts at all which in theory should not be possible due to the lack of certain amino acids, the genes found were then also tested in a new yeast 2 hybrid in which there were only 2 known genes, the bait (*Sfrp2* or *Ptn*) and the now known library construct gene. If the interaction could be confirmed by this second yeast 2 hybrid screen, the gene was classed as a possible candidate for interaction, Figure 8.



Figure 8. Second Yeast 2 hybrid screen confirming the interaction between *Ptn* and the two genes found in the earlier library screen: *Ap1g2* and *Atp5a1*. Colonies that respond to the X- α -Gal, with which the plate was pre-treated, by secreting α -galactosidase turned visibly blue and was another criteria for interaction.

All in all, five probable interaction partners emerged for *Ptn* in these screens, 3 independent screen for both *Ptn* and *Sfrp2*, with *Anapc2* and *Ap1g2* being somewhat more likely due to appearing more than once, but the screens does not allow for any assumptions regarding the quality of these possible, for they are still just possible, interactions which leads me to list them all here in Table 3.

Yeast Two-Hybrid screen: Lin-Kit+ Library against Ptn							
Gene	Screen	Amp Clone	GenBank	start	end	size	Identity
<i>Anapc2</i>	1	1,3,7,10	NM_175300.3	1391	2241	824	99%
<i>Ap1g2</i>	1, 2	6, 8, 1-17, 4-12, 7-13, 4-14,	NM_001110795.1	1428	2132	705	99%
<i>Atp5a1</i>	1	5	NM_007505.2	1095	1759	665	100%
<i>Pias3</i>	1	14	NM_018812.1	797	1510	714	99%
<i>Sgol1</i>	1	4	NM_028232.1	542	981	440	100%

Table 3. Ptn yeast 2 hybrid. Showing the genes, the number of times the gene was found, which screen it was found and sequences identity of possible interaction partners of *Ptn* in 3 independent Yeast 2 hybrid screens.

Some attempts were made to further prove these interactions by using several “pull down” techniques, which would in theory precipitate Ptn along with its interaction partner/s. This was done by taking the plasmids, GAD and GBK, from the Yeast 2 Hybrid, containing the *Ptn* sequence and the sequences found to interact, and using them as a template for protein synthesis with the TNT[®] Quick Coupled Transcription/Translation System (Promega). The synthesised proteins could then be separated in “pull down” experiments due to the c-Myc tag and hemagglutinin (HA) tag that comes along with the expression on the GBK and GAD plasmids respectively. However, these studies proved to be inconclusive (data not shown).

For *Sfrp2* the number of genes found to be interacting with it was somewhat higher, but proved to be mostly made up by ribosomal proteins which are mostly expressed at very high levels in all cells making these interactions rather unlikely, albeit not impossible. Two of the genes identified here, *Vav1* and 2210010N04Rik did show up in two independent screens and due to the involvement of *Vav1* in hematopoiesis and cancer (Gu et al., 2006) it seemed like a very promising candidate. However, the common sequences found in the two screens was shown to be outside the reading frame of *Vav1* making it quite likely that the interaction was more due to chance than a real in vivo interaction. The genes identified in the *Sfrp2* Yeast 2 hybrid screens can be found in Table 4.

Yeast Two-Hybrid screen: Lin-Kit+ Library against <i>Sfrp2</i>							
(41 clones analysed, 17 clones (41%) without identifiable insert)							
Gene	Screen	Amp Clone	GenBank	start	end	size	identity
<i>Vav1</i>	1	36	NM_011691.3	2255	2870	615	99
	1	90	NM_011691.3	2243	2866	623	99
	3	Hefe11	NM_011691.3	2757	2870	113	100
<i>2210010N04Rik</i>	1	14	NM_027410.1	2951	3884	933	95
	1	60	NM_027410.1	2950	4071	1121	92
<i>Rplp1</i>	2	H1A1	NM_018853.3	210	466	256	
	3	Hefe1	XM_001479214.1	242	529	287	97
<i>Rpl35</i>	2	H10A1	XM_001471982.1	59	487	428	98
	2	H20A1	XM_001471982.1	59	486	427	98
<i>Rps11</i>	2	HA11A1	NM_013725.2	358	635	277	99
	2	H15A1	NM_013725.2	358	635	277	98
<i>Actb</i>	3	Hefe7	BC040513.1	9	494	485	99
<i>Hmha1</i>	2	H18A1	NM_027521.2	2601	3477	876	99
<i>Lyl1</i>	2	H7A1	NM_008535.2	498	1513	1015	95
<i>Mitf3</i>	3	Hefe8	NM_029581.3	1167	1558	391	99
<i>Rpl13a</i>	2	H20A1	NM_009438.4	443	655	212	
<i>Rps19</i>	3	Hefe10	NM_023133.1	458	629	171	98
<i>Tmem160</i>	2	H3A1	NM_026938.1	467	840	373	
<i>Tor2a</i>	1	59	NM_152800.2	662	1495	833	99
<i>Zc3hc1</i>	2	H4A1	NM_172735.2	1746	1847	101	100
<i>Zdhhc16</i>	2	H4A2	NM_023740.1	711	1762	1051	98
<i>Zfp51</i>	1	87	NM_001007568.2	2099	3141	1042	93

Table 4. *Sfrp2* yeast 2 hybrid. Showing the genes, the number of times the gene was found, which screen it was found and sequences identity of possible interaction partners of *Sfrp2* in 3 independent Yeast 2 hybrid screens.

3.5 Discussion

The ClonTech Atlas Mouse arrays paved way for the subsequent Yeast 2 hybrid screens by allowing me to select some starting points for this project, which were the genes *Ptn* and *Sfrp2*. As previously mentioned these genes were selected on a basis of expression data but also what was already know about the genes, for example *Ptn* was known to be a quite small secreted protein involved in a number of different processes in vivo and *Sfrp2* became very interesting in its proposed role acting as an inhibitor of canonical Wnt signalling which was then emerging as a very important

pathway in the regulation of hematopoiesis. So if it was possible to find the interaction partners of these genes in the HSC it could very well lead to potential clinical uses to combat hematopoietic disorders if such an agent could be administered via the blood stream for example. That notion prompted the following study which was to try and identify those interaction partners using the Yeast 2 hybrid system acquired from CloneTech.

The use of a Yeast 2 hybrid screen has been proven before to be able to find interaction partners of the genes tested. While this is the case for some genes it did not seem to be so in ours. One of the known hazards concerning this was the fact that this is a yeast based system in which it is yeast cells and not mammalian cells that are responsible for the production of the proteins that should later interact with each other. This will more than likely result in more than just slight post-translational alterations to the proteins compared to the wild type mammalian one. While this might be less of an issue for nuclear proteins it is of fundamental importance if looking for interaction partners for proteins that are supposed to be secreted, and since all the interactions must take place in the yeast nucleus none of these alterations should possibly be present resulting in a truly artificial situation which has little resemblance to the real situation in vivo. One example that is pointing to this possibility is the *Vav1* sequence which interacted with the *Sfrp2* derived peptide, but in the end turned it out that the sequence found in the interacting plasmid was found outside the actual reading frame for *Vav1*, making it highly unlikely to be a real interaction in vivo. Further experiments with the proposed candidates to interact with Ptn (*Sgol1*, *Anapc2*, *Ap1g2*, *Atp5a1* and *Pias3*) were carried out by Monica Kröger, who continued the *Ptn* project, but these also proved to be inconclusive with no real proof of interaction (data not shown).

3.4 Materials and methods

Mice

(129xLy5.1)F1 (129Ly5.1) mice were used as cell donors in all experiments. Mice were kept in Microisolators under SPF conditions according to FELASA recommendations.

FACS analysis

Cell suspensions were stained with antibodies in HF2+ buffer for 15 minutes on ice in the dark. For the sorting of hematopoietic progenitor cells the following antibodies were used: phycoerythrin (PE)-labelled Cd117 (c-Kit) and PE-Cy7-labeled Sca1 (Ly-6A/E). Also included was a lineage (Lin) cocktail supplied by Miltenyi (Miltenyi Biotec, Bergisch Gladbach, Germany) in order to remove more mature cells.

The Lin- Cd117+ Sca1+ cells were sorted on a Cell populations were sorted on a MoFlo (Cytomation-Beckman Coulter, Fullerton, CA, USA) supplied with Summit 4.3 software (Beckman Coulter).

mRNA isolation

mRNA was isolated and purified using the RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

Bacteria

DH5alpha bacteria were used in these experiments in order to create the different plasmids that were later inserted into the yeast cells.

Buffers

TB (Transformation buffer)		
PIPES	10 mM	3,021 g/l
CaCl ₂	75 mM	11,025 g/l
KCl	250 mM	18,637 g/l
MnCl ₂	55 mM	10,885 g/l

SOC medium (1l)		
Tryptone	2%	20g
yeast extract	0,5%	5g
NaCl	10 mM	0,58 g
MgCl ₂	2,5 mM	2,5 ml (1M)
MgSO ₄	10 mM	10 ml (1M)
Glucose	20 mM	3,4 g

Vectors

pGADT7 (Clontech) vector for the library constructs and the pGBKT7 (Clontech) vector for the bait proteins (Ptn and Sfrp2).

Yeast strains

Yeast (AH109) handled and maintained according to Clontech manuals.

Library construction

The “genetic library” created from the sorted primitive hematopoietic cells using the SMART™ cDNA Library Construction Kit (Clontech).

Sequencing

Sequencing was carried out by GATC Biotech (Constance, Germany).

Sequences

Reference sequences to ensure the correct product.

Ptn NM_008973.2

```

ATGTCGTCCCAGCAATATCAGCAGCAACGTAGAAAATTTGCAGCTGCCTTCCTGGCATTGATTTTC
ATCTTGGCAGCTGTGGACACTGCTGAGGCCGGGAAGAAAGAGAAACCTGAAAAAAGGTGAAAA
AGTCTGACTGTGGAGAATGGCAGTGGAGTGTGTGCGTGCCTACCAGCGGGGACTGTGGATTGG
GCACCCGGGAGGGGCACTCGCACTGGCGCCGAGTGCAAACAGACCATGAAGACTCAGAGATGTA
AGATCCCTTCAACTGGAAGAAGCAGTTTGGAGCTGAGTGCAAGTACCAGTTCCAGGCTTGGGGA
GAATGTGACCTCAATACCGCCTTGAAGACCAGAACTGGCAGCCTGAAGCGAGCTCTGCACAATG
CTGACTGTCAGAAAAGTGTCAACATCTCCAAGCCCTGTGGCAAGCTCACCAAGCCCAAGCCTCAA
GCGGAGTCAAAGAAGAAGAAAAAGGAAGGCAAGAAACAGGAGAAGATGCTGGATTAA

```

Sfrp2 NM_009144.2

ATGCCGCGGGGCCCTGCCTCGCTGCTGCTGCTAGTCCTCGCCTCGCACTGCTGCCTGGGCTCG
GCGCGTGGGCTCTTCTCTTCGGCCAGCCGACTTCTCTACAAGCGCAGCAACTGCAAGCCCA
TCCCCGCCAACCTGCAGCTGTGCCACGGCATCGAGTACCAGAACATGCGGCTGCCAACCTGCT
GGGCCACGAGACCATGAAGGAGGTGCTGGAGCAGGCGGGCGCCTGGATTCCGCTGGTCATGAA
GCAGTGCCACCCGGACACCAAGAAGTTCTGTGCTCGCTCTTCGCCCTGTCTGTCTCGACGAC
CTAGATGAGACCATCCAGCCGTGTCACTCGCTCTGCGTGCAGGTGAAGGACCGCTGCGCCCCG
GTCATGTCCGCTTCGGCTTCCCCTGGCCAGACATGCTGGAGTGCGACCGTTTCCCGCAGGACA
ACGACCTCTGCATCCCCCTCGCTAGTAGCGACCACCTCCTGCCGGCCACAGAGGAAGCTCCCAA
GGTGTGTGAAGCCTGCAAAACCAAGAATGAGGACGACAACGACATCATGGAAACCCTTTGTAAAA
ATGACTTCGCACTGAAAATCAAAGTGAAGGAGATAACGTACATCAACAGAGACACCAAGATCATC
CTGGAGACAAAGAGCAAGACCATTTACAAGCTGAACGGCGTGTCCGAAAGGGACCTGAAGAAAT
CCGTGCTGTGGCTCAAAGACAGCCTGCAGTGACCTGTGAGGAGATGAACGACATCAACGCTCC
GTATCTGGTCATGGGACAGAAGCAGGGCGGGCAGCTGGTGATCACCTCCGTGAAACGGTGGCA
GAAGGGCCAGAGAGAGTTCAAGCGCATCTCCCGCAGCATCCGCAAGCTGCAATGCTAG

PCR

For PCR a peqGold PCR –Mastermix Y was used (PeqLab, Erlangen, Germany).

Primers

Gene	RefSeq	Forward	Reverse
<i>Sfrp2</i>	NM_009144.2	ATGCCGCGGGGCCCTGCC	CTAGCATTGCAGCTTGCGGA
<i>Ptn</i>	NM_008973.2	GCTGCCTTCCTGGCATTGAT	TTGCCACAGGGCTTGGAGAT

Yeast 2 hybrid screen

The yeast 2 hybrid screen was carried out according to Clontech instructions. First a primary screen with only two amino acids missing (Leu, Trp), then followed by further screening using plates missing up to 4 amino acids and also screening for the expression of *LacZ*, indicated by blue colonies.

Cloning

Sfi1 digestion sites (GGCCNNNNNGGCC) added to primer sequences for the generation of suitable inserts into the two vectors (GAD, GBK). Insert was ligated into the plasmid using the Quick ligationTM (New England Biolabs, Ipswich, MA, USA)

Sequence analysis

Sequences obtained were analysed using the BLAST software at the NCBI homepage (<http://www.ncbi.nlm.nih.gov/>).

Protein synthesis

Proteins were synthesised using the TNT[®] Quick Coupled Transcription/Translation System (Promega, Madison, USA) which in theory allows for the synthesis of proteins from DNA containing the T7 promotor, something which the GAD and GBK plasmids include.

Western blots (SDS Page)

The following antibodies were used to identify the HA and c-myc tags coupled to the proteins produced with the TNT[®] Quick Coupled Transcription/Translation System (Promega) using the GBK and GAD plasmids with inserts as templates: rabbit anti-HA (C29F4) (New England Biolabs GmbH, Frankfurt am Main, Germany) and anti-c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

Immuno precipitation

Protein A sepharose CL-4B (Amersham Biosciences AB, Uppsala, Sweden) were used in the experiments to find interactions between Ptn and the target genes found in the yeast 2 hybrid screens. Protein A beads were pre-washed 3 times using lysis buffer (1% NP-40, 50mM Tris-HCl (pH 8.0)) then diluted 1:1 with the same buffer and 30µl of this was added to 25µl of the protein mix created with the TNT[®] Quick Coupled Transcription/Translation System (Promega). This new mix was incubated at 4°C for 30 minutes on a shaker and then spun at 14000 rpm using a table centrifuge. Supernatant was moved to a new tube and 2µg of antibodies with which to label it was added, incubated over night at 4°C. The following day: 1 hour on the shaker, 30µl new washed protein A beads added, another hour on the shaker at 4°C, 2 minutes centrifugation at 14000 rpm, removal of supernatant until about 20µl remains, addition of 500µl lysis buffer, samples 1 min on shaker, centrifuged at 14000 rpm for 2 minutes, repeat from addition of 500µl lysis buffer a total of three times, remove as much supernatant as possible, add 25µl loading buffer for western blot, store at -20°C.

Chapter 4: New gene expression array, Affymetrix

4.1 Background

Due to the inconclusiveness of the Yeast 2 hybrid screen and the fact that there were more advanced gene expression array available compared to the ones made prior to my arrival in the lab it was decided to make yet another gene expression array experiment, this time using the GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, USA). These arrays were becoming the golden standard for this kind of expression screens and had a much higher number of probes, 45101, on them compared to the ones described earlier. This would allow us to broaden the search for genes differently expressed by the supporting stromal cell lines (EL08-1D2, UG26-1B6) compared to the ones not able to support long time hematopoiesis (UG15-1B7, AM20-1B4, EL28-1B3 and AM30-3F4). The resulting file containing the expression patterns of these almost 40000 mouse genes was then converted into a database using the FileMaker Pro7 (File maker, Inc). Using this database allowed me to sort out new promising candidate genes to further study the difference between the supporting and non-supporting stroma cell lines. Data obtained from these arrays was also given to collaborating groups which used it as a fundament for further studies which lead to the first publication in Cell Stem Cell (Ledran et al., 2008). The foremost candidate, when comparing the different cell lines, turned out to be Sfrp1, which gave rise to the study that resulted in a second Cell Stem cell publication (Renstrom et al., 2009) showing the important role of Sfrp1 in maintaining hematopoiesis in mice. The data obtained with these arrays will surely yield even more publications in the near future due to the possible applications, quality and the vast amount of data coming out of it.

4.2 Results

After proper analysis the data from these arrays showed the expression patterns of more than 45000 different genetic probes, including most known mouse genes but also many possible genes not yet full investigated. The information was then converted into a database using the Fila Maker Pro7 (Filemaker, Inc) software. Using the statistical an organisational tools available in the Microsoft Excel (Microsoft, Redmond, USA) it was possible to arrange the data in such a way that finding differently expressed genes, be it over- or under expressed, was made easy and a snapshot of this can be found in Figure 9.

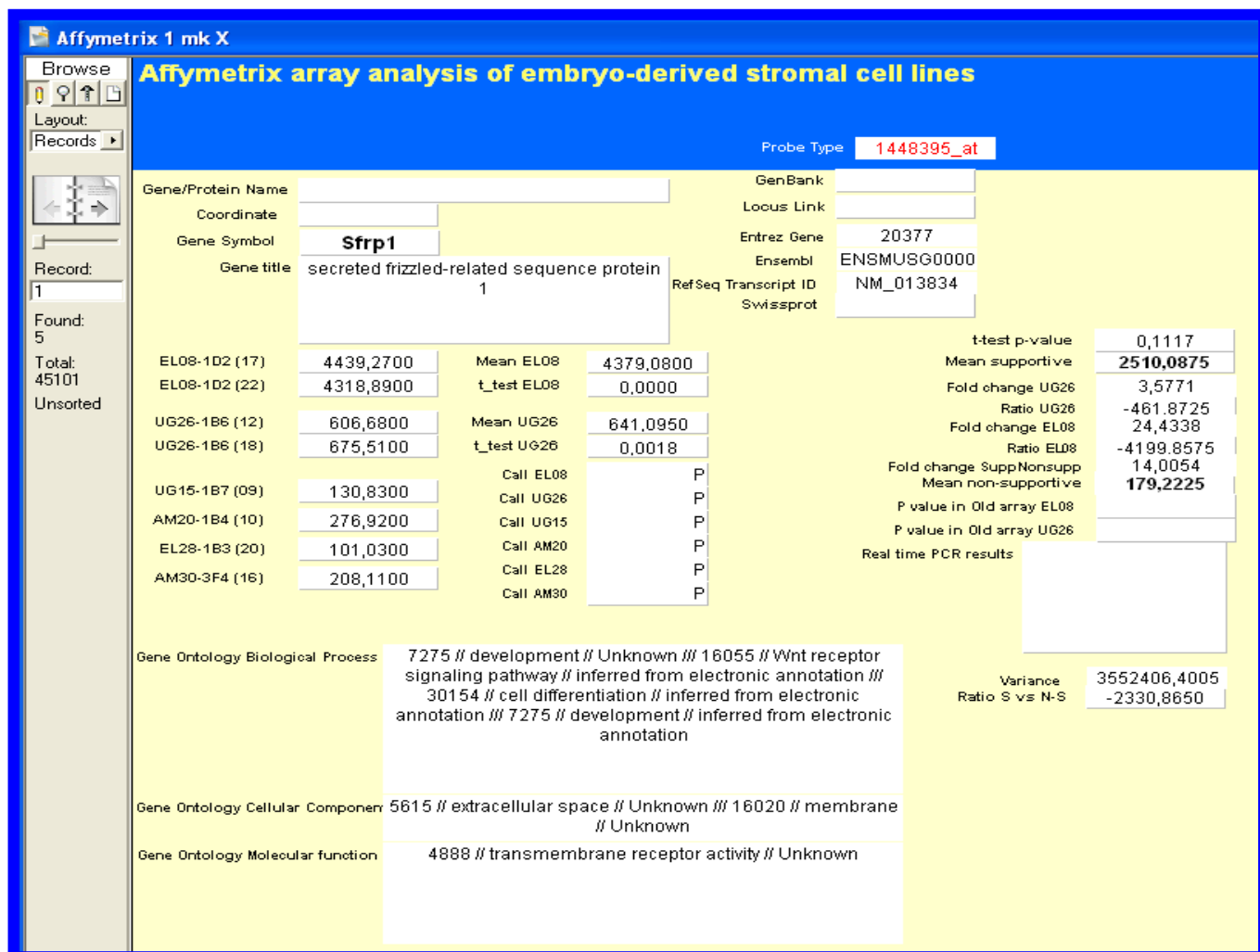


Figure 9. New databases based on the Affymetrix arrays. A snapshot of the database containing the gene expression data of the stroma cell lines, shown here is the profile for *Sfrp1*

A list containing the genes found in the database to match the following criteria: an expression over 200 in both UG26 and EL08, >2 fold change difference between both UG26 and EL08 compared to non-supporting cell lines (UG15-1B7, AM20-1B4, EL28-1B3 and AM30-3F4) and a p-value <0,01 when it comes to expression differences between the supportive and non-supportive lines, can be found in Table 5. Out of the genes that were found to be significantly different in the supportive and non-supportive cell lines, the *Sfrp1* gene, shown above, was found to be the most interesting and worthy of further studies. The reasons for this were several such as it being involved in Wnt signalling, being a secreted protein, the degree of over expression and because of *Sfrp2* being found in the older array made in 2005 (Oostendorp et al., 2005).

	Gene	Ref Seq	Expression	Fold change	P-value
EL08-1D2	<i>Sfrp1</i>	NM_13834	4379	24,4	0,0000005
UG26-1B6	<i>Sfrp1</i>	NM_13834	641	3,58	0,0018
	Gene	Ref Seq	Expression	Fold change	P-value
EL08-1D2	<i>Serpina3g</i>	XM_484175	2878	8,23	0,0009
UG26-1B6	<i>Serpina3g</i>	XM_484175	2759	7,89	0,0011
	Gene	Ref Seq	Expression	Fold change	P-value
EL08-1D2	<i>Dscr111</i>	NM_030598	756	7,57	0,0001
UG26-1B6	<i>Dscr111</i>	NM_030598	383	3,83	0,0009
	Gene	Ref Seq	Expression	Fold change	P-value
EL08-1D2	<i>Gpx7</i>	NM_024198	549	4,67	0,0025
UG26-1B6	<i>Gpx7</i>	NM_024198	546	4,64	0,0026
	Gene	Ref Seq	Expression	Fold change	P-value
EL08-1D2	<i>Olfml3</i>	NM_133859	620	2,8	0,0094
UG26-1B6	<i>Olfml3</i>	NM_133859	645	2,91	0,0075
	Gene	Ref Seq	Expression	Fold change	P-value
EL08-1D2	<i>C1galt1</i>	NM_052993	403	4,8	0,0003
UG26-1B6	<i>C1galt1</i>	NM_052993	222	2,65	0,0005
	Gene	Ref Seq	Expression	Fold change	P-value
EL08-1D2	<i>Slc29a1</i>	NM_022880	376	2,74	0,0005
UG26-1B6	<i>Slc29a1</i>	NM_022880	394	2,87	0,0003
	Gene	Ref Seq	Expression	Fold change	P-value
EL08-1D2	<i>Nudt21</i>	NM_026623	425	2,14	0,0037
UG26-1B6	<i>Nudt21</i>	NM_026623	408	2,05	0,0048

Table 5. List of genes from the new gene expression arrays. Genes found to be significantly over expressed in the affymetrix array, according to the criteria described above.

4.3 Discussion

The GeneChip® Mouse Genome 430 2.0 Array is a superior tool to use compared to the older ClonTech Atlas Mouse 1.2 and 1.2II used prior to my arrival, mainly due to the easier and safer handling of the arrays, radioactivity was needed in the ClonTech arrays while this is not the case with the Affymetrix arrays, but mainly due to the fact that there were only some thousand genes on the old arrays and more than 40000

on the new. This increased the scope of the investigation more than ten fold and by doing so maybe also increased the chance of finding important molecules that might not have been present in the first arrays at all. Out of the top 8 genes (*Sfrp1*, *Serpina3g*, *Dscr111*, *Gpx7*, *Olfml3*, *C1galt1*, *Slc29a1* and *Nudt21*) found in this array using the criteria described above yielded some very interesting molecules, and out of these *Sfrp1* was selected for further study. This turned out to be a very good choice even though the other interesting genes that have not been investigated yet that might shed even more light over the mechanisms that dictate hematopoiesis. For example a study by Mizukami and co-workers in 2008 showed *Serpina3g* to be a gene highly expressed by HSCs and also found to be expressed in the trabecular bone region in the bone marrow, the supposed site of the bone marrow HSC niche (Mizukami et al., 2008). This if course makes sense if the supportive cell lines in this study are mimicking the bone marrow stroma cells that support hematopoiesis, however, this find came years after deciding on which gene to follow further but can be used to further validate the results found here. The resulting study which came out of all this data, using mice deficient in *Sfrp1*, showed that there was in fact a great impact of *Sfrp1* in hematopoiesis, on the maintenance of stem cells and their progeny.

So in short, one can argue that using these cell lines (UG26-1B6 and EL08-1D2), as a stand in for the actual cells making up the HSC bone marrow niche, indeed has merit. This due to the role in hematopoiesis found in several of the genes identified using this method and more could very well be investigated in the future to reveal even more of the complex hematopoietic machinery. The data can today be publicly accessed through the Gene Expression Omnibus; accession number GSE11589, Figure 10.

NCBI logo | GEO Gene Expression Omnibus

HOME | SEARCH | SITE MAP | Handout | NAR 2006 Paper | NAR 2002 Paper | FAQ | MIAME | Email GEO

NCBI > GEO > **Accession Display** [?](#) Not logged in | [Login](#) [?](#)

Scope: Format: Amount: GEO accession:

Series GSE11589 [Query DataSets for GSE11589](#)

Status: Public on Jul 07, 2008

Title: Gene expression analysis of embryo-derived stromal cell lines

Organism(s): [Mus musculus](#)

Experiment type: Expression profiling by array

Summary: Hematopoiesis occurs in a microenvironment in which stromal cells are prominent. Stromal cells have been shown to maintain stem cell behaviour of hematopoietic stem cells. We derived several different stromal cell lines from midgestation embryos which will, or will not maintain hematopoietic stem cells in cultures. We used microarrays to detail the global programme of gene expression underlying maintenance and emergence of hematopoietic stem cells in cocultures with embryo-derived stromal cells.

Keywords: Comparison
CEL files will become available on November 29, 2008

Overall design: Embryo-derived stromal cell lines were grown to confluence and irradiated as described earlier (Oostendorp et al., *Stem Cells*. 2005;23:842:50). Normalized Gene expression of the three different cell lines was compared.

Contributor(s): [Renström J](#), [Mages J](#), [Judex M](#), [Lang R](#), [Oostendorp RA](#)

Citation(s): Ledran MH, Krassowska A, Armstrong L, Dimmick I et al. Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches. *Cell Stem Cell* 2008 Jul 3;3(1):85-98. PMID: [18593561](#)

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Figure 10. Snapshot from Gene Expression Omnibus: accession number GSE11589.

4.4 *Sfrp1*

The family of secreted frizzled-related proteins (*Sfrp*) was first described by Finch et al in 1997 (Finch et al., 1997), and caught their attention by the homology of the so-called frizzled region of the protein usually found in Frizzled proteins, a tell tale sign which would identify them as involved in Wnt signalling. In mouse and humans the

family consists of five members (Kawano and Kypta, 2003), *Sfrp1-5*, and divided into two subfamilies, based on sequence similarities, with *Sfrp1*, *Sfrp2* and *Sfrp5* in one group and *Sfrp3* and *Sfrp4* in the other (Jones and Jomary, 2002). The members of the *Sfrp1* sub-family share a distinct homology in their cysteine rich domain (CRD) and are able to inhibit canonical Wnt signalling (Suzuki et al., 2004) which is one of the main reasons the *Sfrp1* was chosen to be the gene of interest in these studies. It has been suggested that *Sfrp1* interacts with Wnt1 (Dennis et al., 1999), 2 (Xu et al., 1998), 3a (Galli et al., 2006), 5a (Matsuyama et al., 2009), and 7b (Rosso et al., 2005), but some of these findings are based on homology and also not carried out in the murine system. For example, Dennis et al., 1999, claims that *Sfrp1* interacts with Wnt1 but not Wnt5a while a more recent study by Matsuyama et al., claims that *Sfrp1* does interact with Wnt5a after all. Even though all papers state that *Sfrp1* itself acts as an inhibitor of Wnt signalling, by either direct interaction with Wnt proteins or possibly directly on the Fzd receptors themselves, there are also claims that *Sfrp1* works as an agonist at lower concentrations (Uren et al., 2000), but the role of *Sfrp1* does not limit itself to that and has also been described to also stimulate the non-canonical pathway. This could of course have some interesting implications which will be discussed at a later stage in this thesis. *Sfrp1* with its influence on Wnt signalling, be it antagonistic, agonistic, canonical or non-canonical, is involved in many processes in the body, such as anteroposterior axis elongation in the embryo (Satoh et al., 2006), male sexual development in mice (Warr et al., 2009), bone formation (Bodine et al., 2004; Hausler et al., 2004), wound healing (Li and Amar, 2006), methylation of *Sfrp1* is common in acute lymphoblastic leukemia (Roman-Gomez et al., 2007) and probably many more functions in the body. What in the end made it very interesting for this study was the association with bone formation which has a large impact on the conformation of the HSC bone marrow niche and later the involvement in leukemia.

Mouse Secreted frizzled-related protein 1, *Sfrp1*, is located on chromosome 8, contains a 4351 bp transcript resulting in a roughly 35 kDa secreted protein. The protein contains two identified domains called the netrin domain and the frizzled domain, Figure 11.

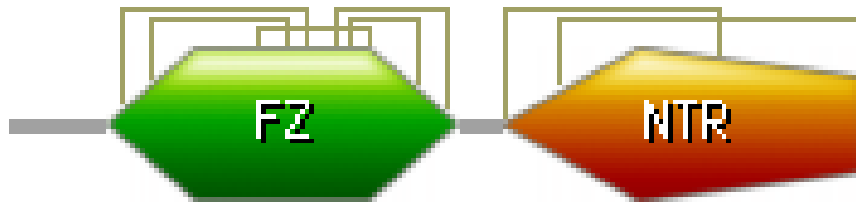


Figure 11. Domain structure of *Sfrp1*. FZ stand for Frizzled, and is the so called cystein rich domain (CRD), which shares sequence homology with the ones found in Frizzled proteins, NTR stands for Netrin domain and is associated with inhibitory effects. Taken from the www.expasy.ch/prosite/ website.

4.5 Materials and methods

Cell lines and cell culture

The embryo-derived stromal cell lines EL08-1D2, UG26-1B6, UG15-1B7, AM20-1B4, EL28-1B3, and AM30-3F4 were cultured as described previously (Oostendorp et al., 2002). Cells were grown on 15 cm cell culture plates (TPP, Trasadingen, Switzerland).

mRNA isolation

The isolation of cytoplasmic RNA was carried out using the RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. 10µg of cytoplasmic RNA was used for the array.

Array readings

The mRNA was applied to the arrays and analyzed on a GeneChip Scanner 3000 7G, enabled for High-Resolution Scanning (Affymetrix) using the GeneChip Operating Software (GCOS) v1.1.1 software, this was carried out by Jörg Mages and Roland Lang at the Department of Microbiology and Immunology, Technical University Munich, Germany.

Array analysis

The data obtained from the GeneChip Scanner 3000 7G was converted into Microsoft Excel (Microsoft) files, which could then be used in the File Maker Pro7 (Filemaker, Inc) to construct databases in which searching for different criteria, such

as expression levels and other statistics, was made easily available to make the best use of the very large amount of data produced.

Statistical analysis

Unpaired students T-test was used for the statistical analyses.

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Chapter 5: Secreted frizzled-related protein 1 (*Sfrp1*) extrinsically regulates cycling activity and maintenance of hematopoietic stem cells

Cell stem cell

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5.1 Summary

Secreted frizzled-related protein 1 (*Sfrp1*) is highly expressed by stromal cells maintaining hematopoietic stem cells (HSC). *Sfrp1* loss in stromal cells increases production of hematopoietic progenitors, and, in knockout mice, dysregulates hemostasis, and increases Flk2- CD34- Lin- Sca1+ Kit+ (LSK) cell numbers in bone marrow. Also, LSK and multipotent progenitors (MPP) resided mainly in the G0/G1 phase of cell cycle, with an accompanying decrease in intracellular β -catenin levels. Gene expression studies showed a concomitant decrease *Ccnd1* and *Dkk1* in Cd34- LSK cells, and increased expression of *Pparg*, *Hes1* and *Runx1* in MPP.

Transplantation experiments showed no intrinsic effect of *Sfrp1* loss on the number of HSC or their ability to engraft irradiated recipients. In contrast, serial transplantations of wild-type HSC into *Sfrp1*^{-/-} mice show a progressive decrease of wild-type LSK and MPP numbers. Our results demonstrate that *Sfrp1* is required to maintain HSC homeostasis through extrinsic regulation of β -catenin.

5.2 Introduction

Hematopoietic stem cells (HSC) reside in a heterogeneous microenvironment (or niche), in which they generate all mature blood cells. The niche is made up of several different cell types: hematopoietic cells at different stages of differentiation, vascular cells, bone-lining cells as well as neural cells which tightly regulate the self-renewal capacity and multipotency of HSC (Morrison and Spradling, 2008). The microenvironment permits the maintenance of HSC for the entire lifetime of an organism. Moreover, the microenvironment is an efficient sensor of cellular stress, which allows flexible responses to maintain the steady-state of the blood cell system. The precise mechanisms of HSC maintenance are still not fully understood.

Prominent players in the regulation of self-renewal and differentiation are the lipid-modified Wnt-signalling members. The Wnt family members bind frizzled (Fzd) receptors to activate downstream signalling in catenin-dependent canonical and non-canonical pathways. In the canonical pathway Fzd associates with the Lrp5/6 receptor, and signals are propagated through catenins to activate Tcf/Lef transcription complexes. The level of catenins is regulated through serine and threonine phosphorylation and marked for degradation by the Skp1/Cul1/beta-TrCP ubiquitin ligase complex. Activation of catenin-dependent signalling regulates genes

involved in cell cycle regulation and proliferation such as *Ccnd1*, *c-Myc*, but also *Spp1*, *Socs2*, *P2ry14*, and many others (Nygren et al., 2007). In non-canonical signalling Fzds associate with either Ryk or Ror receptor tyrosine kinases to activate calmodulin/Ca²⁺-, or Rho-dependent responses, which, in turn, activate Nfat or the Jun-dependent AP-1 complex, respectively. These pathways regulate a different set of Wnt targets, such as *Pparg* and *Pcdh8* (Takada et al., 2007). Also, there is cross-talk between non-canonical and canonical pathways, as Ca²⁺-dependent signals inhibits catenin stability through Camk2-mediated activation of Nemo-like kinase (Nlk) and subsequent phosphorylation of catenin (Ishitani et al., 2003).

Knowledge about specificity of Fzds and Wnts for canonical or non-canonical pathways is limited. To complicate matters, there is a range of different Wnt-signalling inhibitors such as *Dkk*, *Wif* or *Sfrp*'s, or other Wnt antagonists, such as *Kremen1*, *Ctgf*, *Cyr61*, *Sost* and *Sostdc1*. Paradoxically, some of these directly stimulate certain Fzds independent of Wnt factors. For instance, *Sfrp1* directly activates Fzd2 (Rodriguez et al., 2005), as well as Fzd4, and Fzd7, (Dufourcq et al., 2008). Also, Wnt-factors have been suggested to initiate Fzd-independent signalling events. For instance, *Wnt5a* activates Fzd-independent signalling through both Ror (Fukuda et al., 2008) and Ryk (Keeble and Cooper, 2006).

Definitive HSC emerge from the AGM region between E10 and E11. During this midgestational transition, a significant up regulation of β -catenin (*Ctnnb1*) is observed (Orelia and Dzierzak, 2003). In addition, over expression of *Ctnnb1* results in expansion of the HSC pool, but, at the same time, to loss of myelopoiesis (Kirstetter et al., 2006; Scheller et al., 2006), suggesting that *Ctnnb1* regulates self-renewal and inhibits differentiation. Surprisingly, conditional deletion of *Ctnnb1* or both *Ctnnb1* and *Jup* does not affect the repopulating ability of HSC (Cobas et al., 2004; Jeannet et al., 2008; Koch et al., 2008). Taken together, these results suggest that canonical Wnt signalling may be important in definitive hematopoietic specification, and that catenin stabilization inhibits differentiation and/or lineage commitment.

Non-canonical Wnt signalling has also been shown to play a role in hematopoiesis. Current evidence suggests that this pathway is important in the establishment of the niche, by promoting trabecular bone formation and enlarging endosteal surfaces (reviewed by Yin and Li, 2006), as well as regulating cell cycle of HSC and more mature progenitors. *Wnt5a*, the principal non-canonical stimulator, was shown to

maintain HSC ((Nemeth et al., 2007). Also, the non-canonical intermediate *Camk2* is essential for myeloid proliferation (Si and Collins, 2008). These results show that non-canonical signalling may be important in the control of HSC behaviour. Furthermore, the decrease of *Ctnnb1* after *Wnt5a* stimulation suggests that non-canonical signalling is a natural down regulator of canonical signalling.

In addition to this intrinsic role of Wnt signalling, there is also a role for extrinsic Wnt regulation. For instance, *Wnt3a* stimulates proliferation and inhibits differentiation in co-cultures of marrow cells with stromal cells (Yamane et al., 2001). More interestingly, recently, it was shown that wild-type HSC show increased cell cycling when transplanted into mice transgenic for the canonical inhibitor *Dkk1*, while at the same time the HSC pool is gradually lost (Fleming et al., 2008). Further extrinsic effects were demonstrated in experiments where stromal cells deficient in *Nlk*, were shown to be defective in maintaining hematopoietic progenitors (Kortenjann et al., 2001).

We have isolated a large panel of stromal cell clones from which two cell lines: EL08-1D2 and UG26-1B6, maintain both fetal and adult HSC (Oostendorp et al., 2005; Oostendorp et al., 2002). Comparison of gene expression in these two cell lines with a panel of non-supporting stromal cell lines revealed a number of overrepresented secreted molecules, including Secreted frizzled-related protein 2 (*Sfrp2*) (Oostendorp et al., 2005). We here additionally identify Secreted frizzled-related protein 1 (*Sfrp1*), a secreted 37kDa protein which inhibit canonical Wnt signalling (Kawano and Kypta, 2003), and interact with Wnts 1, 2, 3a, and 7b, as a factor up regulated in both supportive stromal cell lines. Our study shows that *Sfrp1* regulates production of clonogenic cells and long-term HSC maintenance trough an extrinsic signalling mechanism.

5.3 Results

5.3.1 *Sfrp1* is involved in the maintenance of hematopoiesis *in vitro*

Sfrp1, one of the secreted-type of Wnt signalling modulators, was shown to be expressed by bone-lining osteoblast-like cells (Yokota et al., 2008), to regulate skeletogenesis (Hausler et al., 2004) and to inhibit trabecular bone formation in adult mice (Bodine et al., 2004). In hematopoietic malignancies *Sfrp1* acts as a putative tumour suppressor molecule (Huang et al., 2007). We found that *Sfrp1* is up regulated in embryo-derived stromal cells UG26-1B6 (2.2% of Rpl13a) and EL08-1D2 (31.8% of Rpl13a), known to support HSC in non-contact cultures, when compared to cell lines not capable of HSC maintenance (Oostendorp et al., 2005; Oostendorp et al., 2002) (Figure 12a). In order to investigate the role of *Sfrp1* in the maintenance of HSC, we established a stable knock-down of *Sfrp1* of 80% (0.4% of Rpl13a) in the HSC-supportive UG26-1B6 stromal cell line (Figure 12b), which correlates well to the level expressed by the mix of non supportive stroma cell lines. Irradiated knock-down stromal cells (*shSfrp1*) or control cells (pLKO.1) were co-cultured with the lineage negative (Lin⁻) fraction of total bone marrow (BM) cells from control mice in a direct contact manner. Interestingly, Lin⁻ cells cultured on the *shSfrp1* UG26-1B6 generated more colonies compared to the Lin⁻ cells cultured on the control clone (Figure 12c), suggesting that lack of microenvironmental *Sfrp1* promotes hematopoietic progenitor activity *in vitro*.

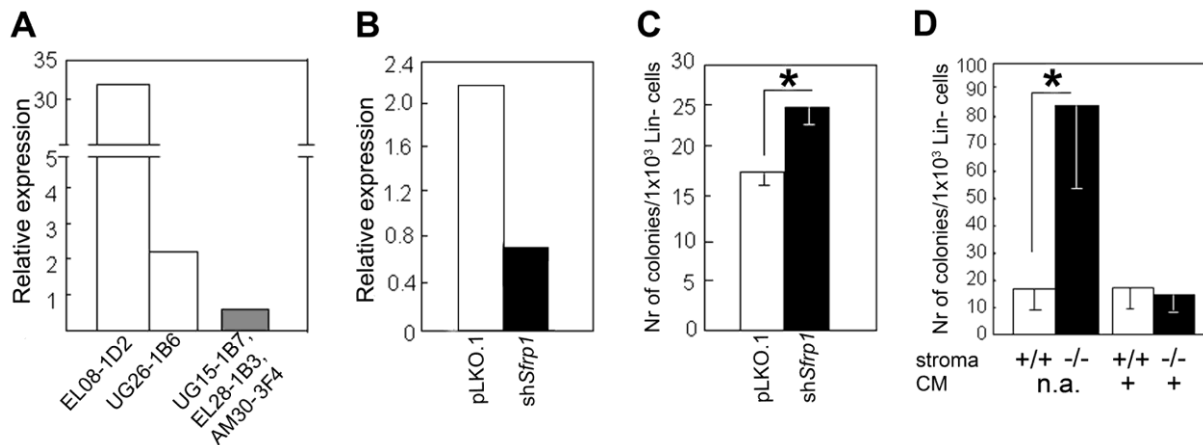


Figure 12. Increased number of wild type progenitors after being co-cultured on *Sfrp1* knock-down and *Sfrp1* knock out stroma.

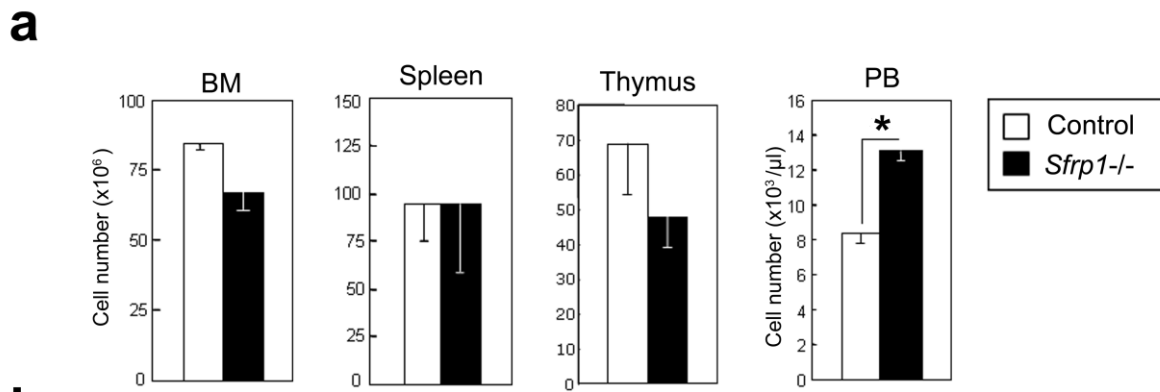
a) The expression of *Sfrp1* in EL08-1D2 and UG26-1B6 cell lines relative to non-supportive stroma (UG15-1B7, EL28-1B3 and AM30-3F4) as measured by real-time PCR. Values were calculated using $2^{\Delta Ct} \times 100\%$ relative to housekeeping gene Rpl13a. **b)** The relative expression (calculated on $2^{\Delta Ct} \times 100\%$ values) of *Sfrp1* in the knock-down clone of UG26-1B6 (sh*Sfrp1*) and in control clone of UG26-1B6 (pLKO.1) relative to Rpl13a. **c)** The number of colonies formed from wild type (wt) Lin- cells after 2 weeks of co-culture on UG26-1B6 sh*Sfrp1* and UG26-1B6 expressing the negative control pLKO1 vector. **d)** The number of colonies formed from wt Lin- cells cultured with primary wt and *Sfrp1*^{-/-} stroma, with and without addition of cell-free conditioned medium (CM) from *Sfrp1*^{+/+} (control) primary stroma. Data shown are the mean number \pm SD of colonies of three to five independent experiments; * = $p < 0,05$.

5.3.2 Absence of *Sfrp1* alters hematopoiesis *in vivo*

To find out how *Sfrp1* affects hematopoiesis, we decided to study the mice deficient in *Sfrp1* (Sato et al., 2006). The knockout mice were originally generated on the 129 background and were paired at least 5 generations with 129B6 background. In contrast to the old *Sfrp1*-deficient mice studied by others (Bodine et al., 2004), the present revealed no gross differences in the extension of the growth plates, but a slight increase in chondrocytes of 10 week old *Sfrp1*^{-/-} mice compared to their controls (Figure S1). As a first experiment we investigated the capacity of primary *Sfrp1*^{-/-} stroma to maintain hematopoiesis using co-cultures with Lin- negative cells, the results were in full accordance with the above findings with the knock-down stromal cell line UG26-1B6 (Figure 12b, 12c). Interestingly, the increased production

of colony-forming cells was completely abolished when the cultures were supplemented by cell-free conditioned medium (CM) from wild-type (*Sfrp1^{+/+}*) stroma, indicating that an Sfrp1-dependent soluble factor was the cause of the increase in hematopoietic progenitors (Figure 12d). However, addition of recombinant Sfrp1, in concentration 1 µg/ml every three days, to cocultures of wt Lin⁻ cells and primary marrow stroma did not affect the generation of hematopoietic progenitors (Figure S2). This suggests that either recombinant Sfrp1 is not biologically active, or that Sfrp1 does not directly act on hematopoietic progenitors. Because of the above results we decided to characterize the hematopoiesis of *Sfrp1^{-/-}* mice in more detail.

The BM, spleen, and thymus cellularity was unchanged (Figure 13a), while the peripheral blood (PB) cell number was significantly increased in *Sfrp1^{-/-}* mice (Figure 13a, 13b). The numbers of eosinophiles (Eos), red blood cells (RBC), hematocrit (HCT) and platelets (PLT) were within normal range (Figure 13b). The main contribution to the increased cell number in peripheral blood come from increased B220⁺ B cell populations (1.4-fold) and Gr1⁺ cells (2.1-fold) (Figure 13c). Also notable was a slight, but significant increase of B220⁺ cells in the BM and thymus, as well as a minor, but significant decrease of both T and B lymphocytes in the spleen (Figure 13c). Taken together, these findings suggest an altered homeostasis of lymphocytes and leukocytes in the absence of Sfrp1.



b

	Control (n = 32)	<i>Sfrp1</i> ^{-/-} (n = 30)	P Value
WBC ($10^3/\mu\text{l}$)	8.38 \pm 0.55	13.12 \pm 0.50	<0.0001
Lymph($10^3/\mu\text{l}$)	6.80 \pm 0.48	10.59 \pm 0.39	<0.0001
Mono ($10^3/\mu\text{l}$)	0.25 \pm 0.02	0.50 \pm 0.03	<0.0001
Gran ($10^3/\mu\text{l}$)	1.31 \pm 0.09	2.04 \pm 0.13	<0.0001
Eos ($10^3/\mu\text{l}$)	0.04 \pm 0.01	0.05 \pm 0.01	NS
RBC ($10^6/\mu\text{l}$)	10.10 \pm 0.19	9.63 \pm 0.11	NS
HCT (%)	54.30 \pm 0.94	51.91 \pm 0.56	NS
PLT ($10^3/\mu\text{l}$)	1156 \pm 100	1104 \pm 52.8	NS

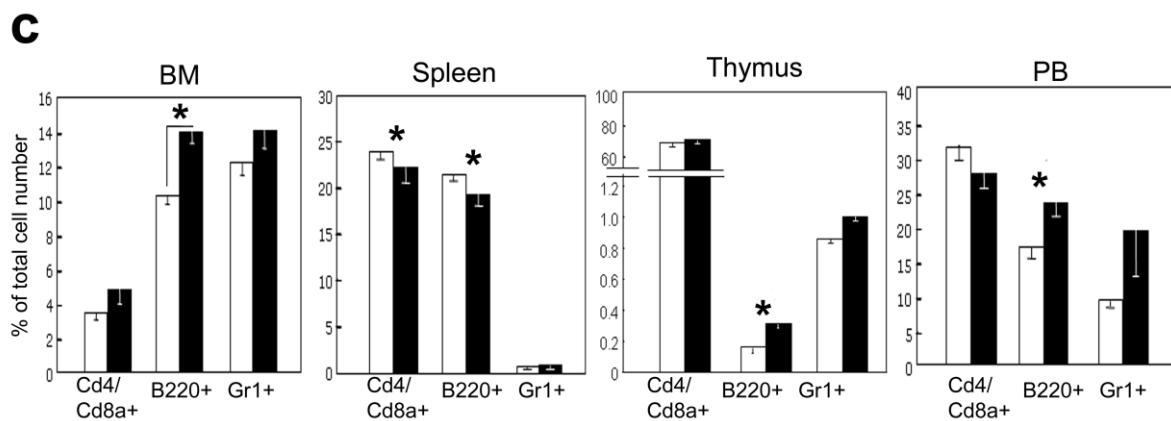


Figure 13. Alterations in steady state hematopoiesis of *Sfrp1*^{-/-} mice.

a) Total cell numbers from bone marrow (BM, four long bones: two femurs and two tibia per mouse), spleen, thymus and peripheral blood (PB, 10^3 cells/ μl). **b)** Blood cell counts in the PB of control (n=32) and *Sfrp1*^{-/-} (n=30). **c)** Total number of Cd4/Cd8a+, B220+ and Gr1+ cells in the BM, spleen, thymus and PB. White bars represent control animals (N=7) and black bars the *Sfrp1*^{-/-} mice (N=5). *Eos*, eosinophiles; *Gran*, granulocyte; *HCT*, hematocrit; *Lymph*, lymphocyte; *Mono*, monocyte; *PLT*, platelets; *RBC*, red blood cell *WBC*, white blood cell; *NS*, not significant. All values are shown as mean \pm SEM. * =p<0,05

To find out whether these changes result from alterations in early hematopoiesis, we analysed the primitive cell populations in multiparameter flowcytometry (Figure 3a). Numbers of Lin⁻, Sca1⁺ and Kit⁺ (LSK) cells were unchanged in *Sfrp1*^{-/-} mice (Figure 14b). However, the number of as the more primitive Flk2 and Cd34 double negative population of LSK (Cd34-Flk2-LSK) (Osawa et al., 1996, Christensen & Weissman, 2001; 1.5-fold, p=0.013 (n=7), Figure 14a, b) as well as Cd150⁺ Cd48⁻ Cd34⁻ Flk2-LSK (Wilson et al., 2008; 1.8-fold, p=0.044 (n=3), Figure S3), were significantly increased in *Sfrp1*^{-/-} mice compared to controls. Further down the hematopoietic hierarchy, we found that multipotent progenitors (Lin⁻, Sca1⁻ and Kit⁺ MPP) were significantly reduced. This, in turn, suggested that the progenitors that make up the MPP population (common myeloid progenitors (CMP), granulocytic progenitors (GMP), and megakaryocytic/erythroid progenitors (MEP)) should be reduced as well, however, we only observed a significant reduction (1.4 fold) in the MEP population (Figure 14c,d). Additionally, the number of common lymphoid progenitors (CLP) in these mice also seemed reduced (1.4-fold), but, due to the intra-animal variance, this difference never reached statistical significance (p=0.12, n=8 for both control and *Sfrp1*^{-/-} groups) (Figure 14c,d). Functionally, the number of colony-forming cells, such as CFU-GEMM was unchanged, but the number of CFU-GM, particularly the large CFU-GM, as well as the BFU-E were significantly decreased (Figure 14e). Thus, loss of *Sfrp1* results in a decreased progenitor cell activity.

5.3.3 Altered cell cycle regulation in the absence of *Sfrp1*

Entry of HSC into cell cycle is regulated by the microenvironment as well as modified by intrinsic factors. Our results suggest that absence of *Sfrp1* affects the production of both progenitors and mature hematopoietic cells. In order to understand which mechanisms underlie these alterations, we studied the cell cycle status of LSK, MPP and mature cells *in vivo*. Animals were injected with BrdU and were sacrificed three

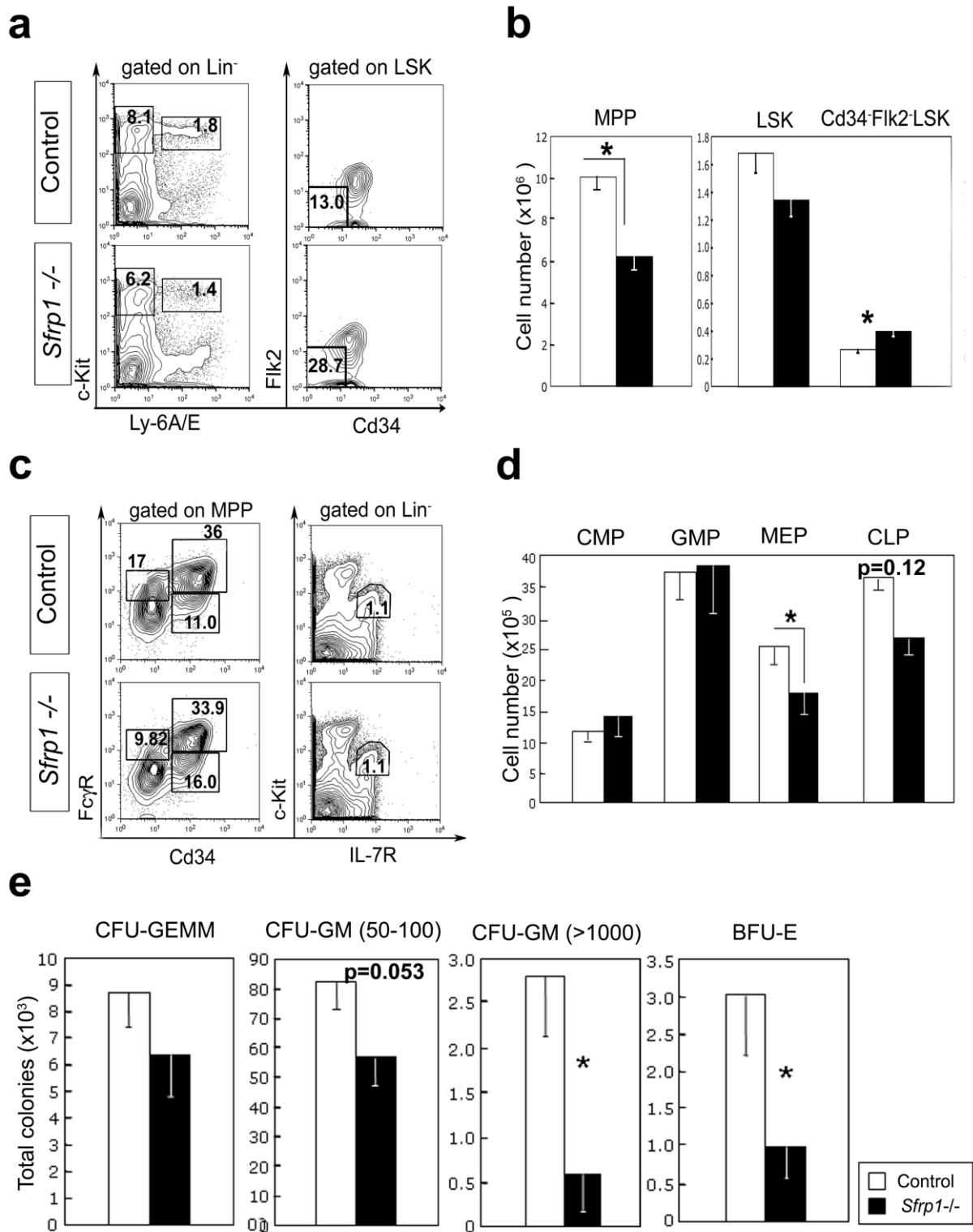


Figure 14. Alterations in early hematopoiesis in *Sfrp1*^{-/-} mice.

a) Representative FACS plots of BM cells isolated from control and *Sfrp1*^{-/-} mice with gates for primitive HSC. **b)** MPP, LSK and Cd34-Fik2-LSK are presented in absolute numbers (N=7). **c)** Representative FACS plots of BM cells isolated from control and *Sfrp1*^{-/-} mice with gates for committed progenitors. **d)** CMP, GMP, MEP and CLP in absolute numbers. (N=8 for control animals and N=6 for *Sfrp1*^{-/-}). **e)** Total number of colonies formed from four long bones (2 femurs + 2 tibia) (N=15 for control and N=11 for *Sfrp1*^{-/-} animals). Open bars represent control and closed bars *Sfrp1*^{-/-}. All values are shown as mean ± SEM. * = p<0,05.

hours later. Bone marrow and blood cells were analyzed for hematopoietic subpopulations (Figure 15a). We found that in the bone marrow, the G0/G1 population is increased in both LSK and MPP cells from *Sfrp1*^{-/-} mice. Correspondingly, a decrease of the S-phase cells was observed, indicating that in *Sfrp1*^{-/-} mice, LSK (p=0.063) and MPP (p=0.012) cycle less (Figure 15b). However, this cycling behaviour changes from early to late hematopoiesis as both mature myeloid Gr1+ cells as well as B220+ cells show an expanded S-phase population in the marrow (Figure 15c). The increase in S-phase was not noticed in Cd4+ or Cd8a+ T cells, indicating that *Sfrp1* does not affect cell cycle of T cells in the marrow (Figure 15c).

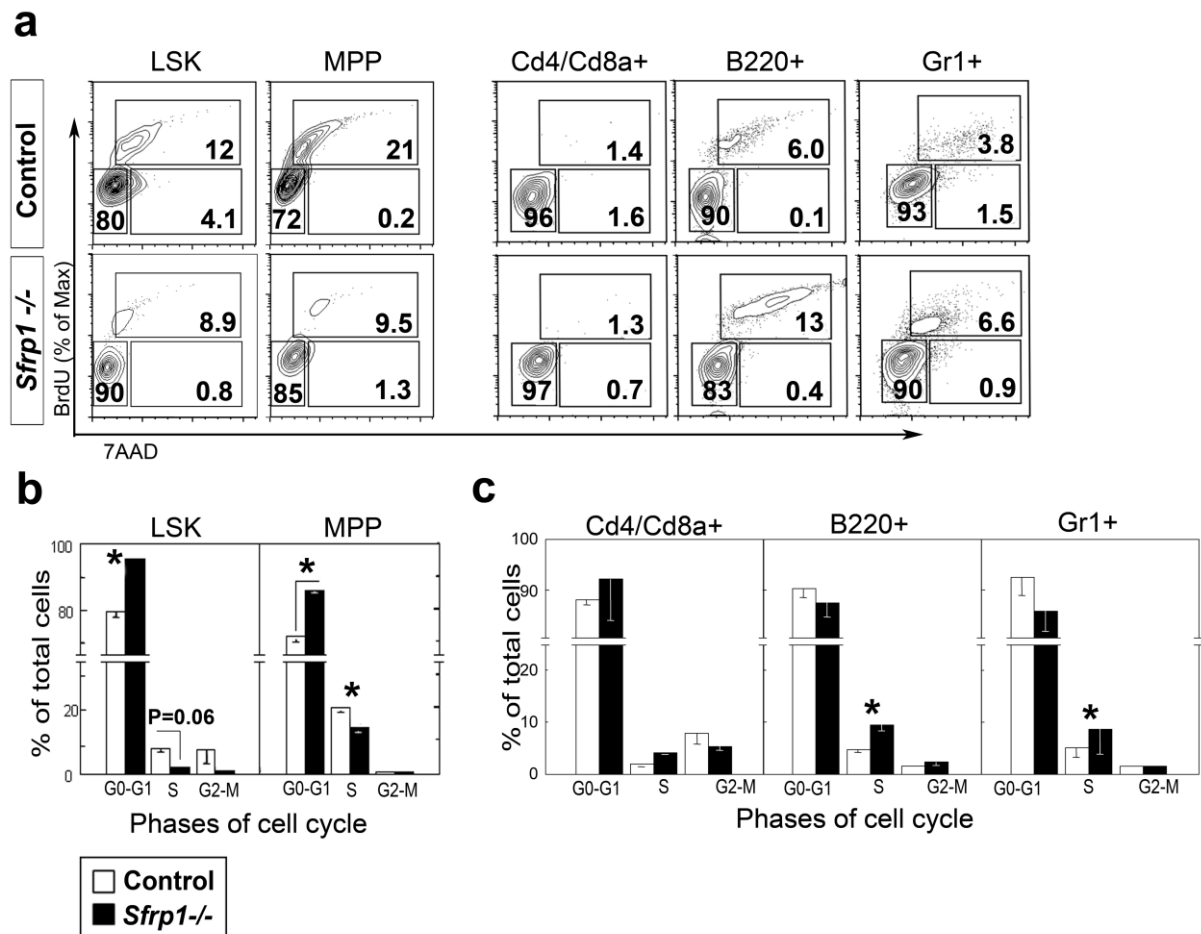


Figure 15. Detection of alterations in cell cycle behaviour in hematopoietic cells from *Sfrp1*^{-/-} mice using in vivo BrdU treatment.

a) Representative FACS plots showing BrdU incorporation in primitive and mature hematopoietic cells isolated from control and *Sfrp1*^{-/-} BM. **b)** Percentage of BrdU positive cells in G0-G1-, S- and G2-M phases of cell cycle in LSK and MPP of control and *Sfrp1*^{-/-} mice. **c)** Percentage of BrdU positive cells in G0-G1-, S- and G2-M phases of cell cycle in T-, B- and myeloid cells of control and *Sfrp1*^{-/-} mice (N=6). All values are shown as mean ± SEM.

5.3.4 Lack of *Sfrp1* alters the expression of signalling molecules in early hematopoietic cells

In order to identify the molecular mechanisms underlying altered hematopoiesis in the absence of *Sfrp1*, we analyzed the expression level of several molecules that could possibly be involved. Intracellular flowcytometric analyses demonstrated that the number of cells expressing high levels of the central canonical Wnt signalling intermediate Ctnnb1 was significantly decreased in LSK, MPP, and CLP of *Sfrp1*^{-/-} mice compared to the control animals (Figure 16a, Figure S4). At the same time P-Ctnnb1, was significantly decreased only in LSK cells of *Sfrp1*^{-/-} compared to the wild type controls (Figure 16a, FigureS4). In contrast, the number of cells expressing *Jnk1* (Mapk8), one of the mediators of non-canonical Wnt signalling, was not altered in *Sfrp1* knock out animals (Figure 16b, Figure S4). To address the more critical question of whether Ctnnb1 accumulates in the nucleus, we performed single cell stains. These stains independently confirmed that Ctnnb1 is present at a lower level in *Sfrp1*^{-/-} LSK, MPP, and CLP (Figure 16c,d). In LSK and CLP control cells, Ctnnb1 was present in clearly visible membrane-bound forms (Figure 16c), whereas in cells derived from *Sfrp1*^{-/-} bone marrow, membrane-bound Ctnnb1 appeared to be absent, resulting in a relatively high nuclear to cytoplasmic Ctnnb1 ratio, particularly in *Sfrp1*^{-/-} LSK cells (Figure 16e). Thus, the difference in nuclear, cytoplasmic Ctnnb1 distribution was clearest in LSK cells (Figure 16e).

To find out how the level and altered distribution of Ctnnb1 affects gene expression, we studied expression levels of “classical” downstream transcriptional targets of the catenin/Tcf/Lef pathway, as well as other molecules mediating Wnt signalling: *Lef1*, *Fzd4*, and *Pparg*. The decreased catenin levels resulted in a significant reduced expression of both catenin targets cyclin D1 (*Ccnd1*) and the Wnt inhibitor dickkopf 1 (*Dkk1*) in Cd34-LSK, but not MPP cells (Figure 16f, Figure S5). The expression of transcription factor *Lef1*, which activates target genes in complex with the nuclear Ctnnb1, was unchanged in Cd34-LSK and MPP of *Sfrp1*^{-/-} mice (Figure 16f, Figure S5). In contrast, the expression of *Pparg*, which is a repressor of Wnt signalling, was significantly up-regulated in MPP (Figure 16f, FigureS5). On the other hand, *Fzd4* expression, which binds *Sfrp1* (Dufourq et al., 2008) and was previously shown to be expressed by HSC (Yokota et al., 2008) was unchanged in Cd34-LSK, but was

significantly decreased in MPPs of *Sfrp1*^{-/-} mice compared to the controls (Figure 16f, Figure S5).

Wnt signalling was shown to affect Notch pathway in HSC (Duncan et al., 2005), and we found a significant up-regulation of the Notch target gene *Hes1* in Cd34-LSK and MPP of *Sfrp1*^{-/-} mice compared to the controls (Figure 16f, Figure S5). This finding is in line with previously published results from transgenic mice over-expressing *Dkk1* (Fleming et al., 2008), another repressor molecule in Wnt signalling. Also the expression of *Runx1*, a downstream mediator of Notch signalling (Burns et al., 2005) was significantly up-regulated in MPPs and not altered in LSK of *Sfrp1*^{-/-} mice (Figure 16f, Figure S5). The NFκB modulator *Ikbkg* was slightly lower expressed in Cd34-LSK cells from *Sfrp1*^{-/-} mice, but unchanged in their MPP (Figure 16f, Figure S5), suggesting possible cross-talk of NFκB and *Sfrp1* signals in the earliest hematopoietic cells. The expression levels of the transcription factor *Lmo2* was unchanged in both populations of *Sfrp1* knock out mice (Figure 16f, Figure S5). Taken together, these results show that the absence of *Sfrp1* alters the expression of both Wnt- and Notch pathway mediators and targets in both MPP and Cd34-LSK.

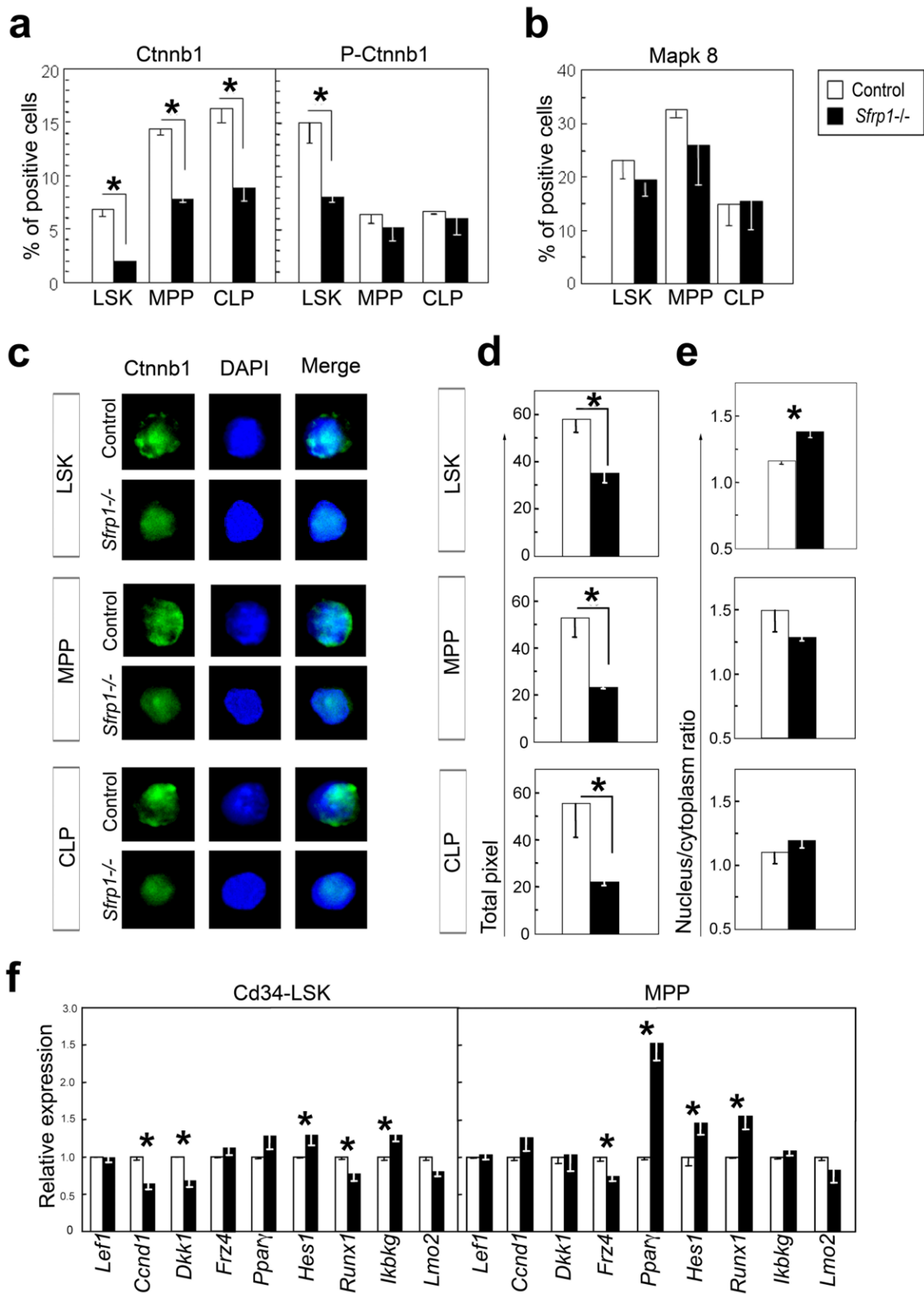


Figure 16. Alterations in β -catenin dependent signalling in *Sfrp1*^{-/-} mice.

a) Intracellular stain of Ctnnb1 and P-Ctnnb1 in LSK, MPP and CLP of control and *Sfrp1*^{-/-} mice (N=3). **b)** Intracellular stain of Mapk8 in LSK, MPP, and CLP. **c)** Single cell stains showing the expression of Ctnnb1 in sorted LSK, MPP and CLP cells (N=3). **d)** Quantification of Ctnnb1 stain (total pixel number) using ImageJ in LSK (N=10), MPP (N=8), and CLP (N=5). **e)** Ratio of nuclear and cytoplasmic Ctnnb1 expression, in sorted LSK, MPP and CLP. **f)** Normalized expression of *Lef1*, *Ccnd1*, *Dkk1*, *Fzd4*, *Pparg*, *Hes1*, *Runx1*, *Ikkbg* and *Lmo2* in Cd34- LSK and MPP of control and *Sfrp1*^{-/-} mice. Results were normalized relative to the combined expression of the three housekeeping genes (*Hprt1*, *Rps21* and *Ythdf1*). All values are shown as mean \pm SEM in 3 to 5 independent experiments* =p<0,05.

5.3.5 The *Sfrp1* deficient microenvironment shows a defect in HSC maintenance

In order to separate intrinsic from extrinsic effects caused by *Sfrp1* loss, we first analyzed engraftment of *Sfrp1*^{-/-} HSC transplanted into 129Ly5.1 recipients. In addition, we performed this experiment in a limiting dilution fashion which would also enable independent functional confirmation of phenotypical results show in Figure 16b. These experiments showed that the number of functional HSC is unchanged in *Sfrp1*^{-/-} mice (Figure 17a). In addition, the pattern of lymphomyeloid engraftment was unchanged with regard to recipients receiving control 129B6 cells (Figure 17b; Figure S6), indicating there are no intrinsic defects in the ability of *Sfrp1*^{-/-} to engraft myeloid and lymphoid lineages.

In order to assess the extent of the extrinsic effect, we analyzed engraftment of wild type Ly5.1 HSC in *Sfrp1*^{-/-} and control 129Ly5.1 mice (primary transplants, 1^o) (Figure 18a). Sixteen weeks after transplantation in 1^o recipients, we observed a significant increase of engrafted wild type cells in BM and PB of *Sfrp1*^{-/-} mice (Figure 18b). The number of mature myeloid cells was increased in spleen and PB of *Sfrp1*^{-/-} mice compared to control (Figure 18c). The maintenance of donor LSK and MPP cells in 1^o *Sfrp1*^{-/-} mice was slightly higher, but not significantly changed (Figure 18d).

a

Limiting dilution	Frequency of positive animals	
	Control	<i>Sfrp1</i> ^{-/-}
150000	3/4	4/6
50000	1/3	2/5
20000	1/5	1/6
Frequency	1 in 107,000	1 in 121,000
Confidence interval	67,000 → 172,000	81,000 → 180,000

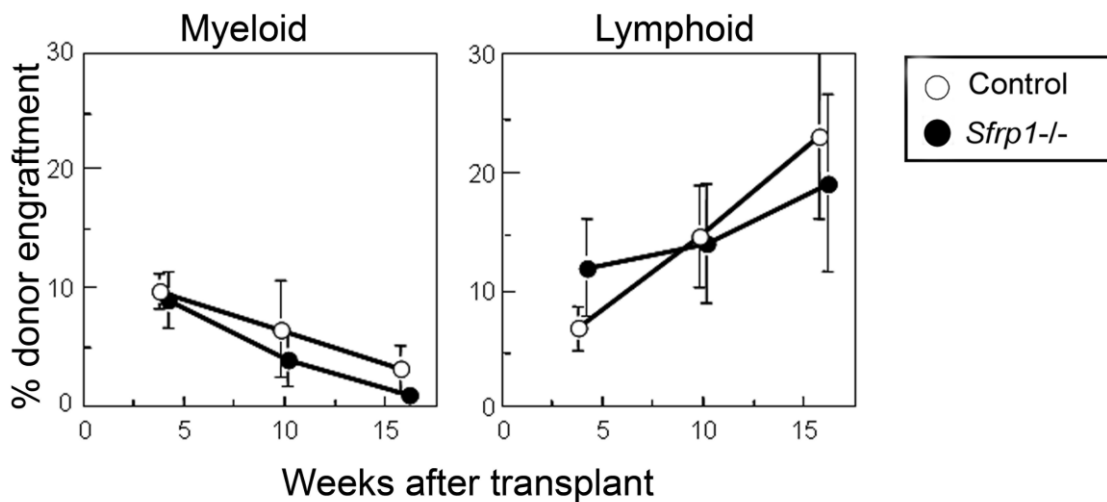
b

Figure 17. No intrinsic defect in the engraftment of *Sfrp1*^{-/-} HSC.

a) Limiting dilution analysis of engrafted cells in the PB of mice transplanted with 150,000, 50,000 or 20,000 of control or *Sfrp1*^{-/-} donor cells. Shown are the number of positive animals per total transplanted as well as the frequency and 95% confidence interval of this frequency determined using the L-Calc software. **b)** The pattern of myeloid and lymphoid engraftment in mice transplanted with 50,000 donor cells. Values are shown as mean ± SEM.

As it was recently shown that mild effects in primary recipients, may, in fact, reflect major and irreversible changes in HSC behaviour and number only observed at later time points (Fleming et al., 2008; Miyamoto et al., 2007), we also performed secondary transplantation (2°). In these experiments we used wild-type recipients, so that any phenotypical changes would depend only on the microenvironment of the 1° recipient. In 2° recipients reconstituted with donor cells from *Sfrp1*^{-/-} 1° mice, we observed decreased total as well as HSC engraftment (Figure 18b, Table S2). Interestingly, we observed a significant increase in the number of B220 positive B cells in all tissues examined - BM, spleen and blood (Figure 18e). In addition, contrary to the 1° *Sfrp1* deficient recipients, the number of donor Gr1+ cells (Figure 18e) as well as LSK and MPP cells were severely decreased in 2° transplants (Figure 18f), indicating that in the primary *Sfrp1*^{-/-} recipients, the ability of the microenvironment to maintain LT-HSC was reduced.

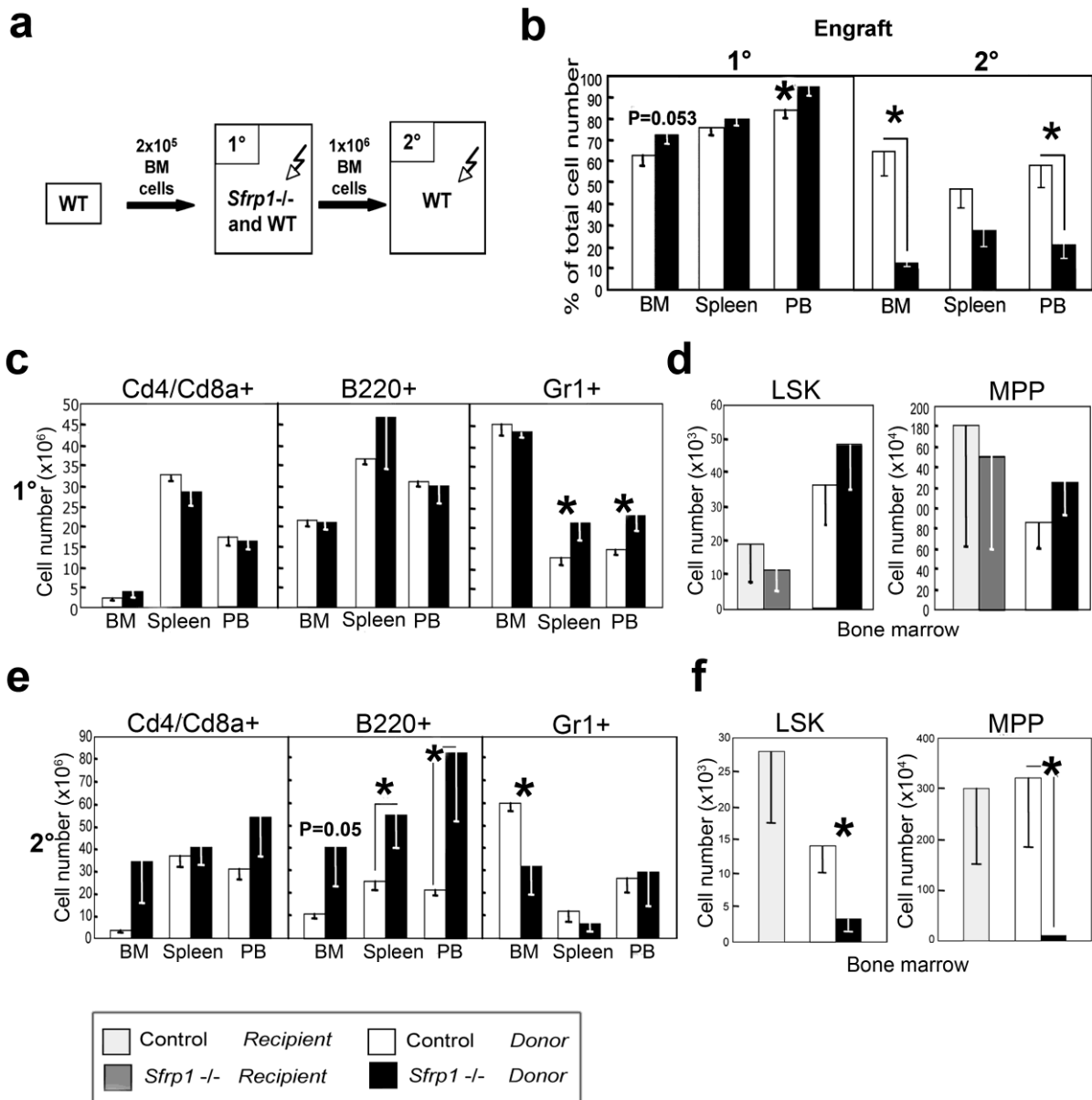


Figure 18. Extrinsic regulation of wild-type HSC engraftment in the *Sfrp1*^{-/-} microenvironment.

a) Serial transplantation flow chart. The wild type BM cells were injected into *Sfrp1*^{-/-} or wild type (*Sfrp1*^{+/+}) recipient mice (1° transplants). 16 weeks post transplantation, BM was isolated and 1x10⁶ BM cells were transplanted into wild type recipients (2° transplants). Transplantations were carried out as three independent experiments each. **b**) The engraftment levels in the BM, spleen and PB of 1° (control (N=9) and *Sfrp1*^{-/-} mice (N=5)) and 2° (cells from 1° BM into N=9 (1° recipient: control), or N=13 (1° recipient: *Sfrp1*^{-/-})). **c**) Absolute numbers of engrafted T (Cd4⁺ and/or Cd8a⁺ cells), B (B220⁺ cells), and myeloid (Cd11b⁺ and/or Gr1⁺) cells in the BM (cells per four long bones), spleen and peripheral blood (PB, cells per μl) of primary recipients **d**) Absolute numbers of engrafted LSK and MPP in the BM (four long bones) of primary recipients **e**) Absolute numbers of engrafted T, B, and myeloid cells in the BM (per four long bones), spleen and PB (per μl) of secondary recipients. **f**)

Absolute numbers of engrafted LSK and MPP in the BM of secondary recipients (per four long bones). All values are shown as mean \pm SEM. * = p <0.05.

5.4 Discussion

In previous studies, we generated embryo-derived stromal cell lines capable of supporting HSC in culture (Oostendorp et al., 2002). By comparing the expression profiles of these cell lines with non-supportive ones, we previously identified up regulation of *Sfrp2* (Oostendorp et al., 2005). In the current study we identified an additional member of Sfrp family: *Sfrp1*, to be overrepresented in HSC-supporting stromal cells. Both, *Sfrp1* and *Sfrp2* are known modulators of Wnt signalling. The members of Wnt signalling pathway have emerged as regulators of HSC self-renewal and proliferation (Murdoch et al., 2005; Reya et al., 2003; Nemeth et al., 2007; Liang et al., 2003; Luis et al., 2008). In particular, the Wnt factors play an important role in the complex interplay of intrinsic signals from HSC and extrinsic stimuli from the surrounding stromal microenvironment, also called the stem cell niche (reviewed in Yin and Li, 2006).

Here, we analyzed the effect of *Sfrp1* on hematopoiesis and found that the down-regulation of *Sfrp1* in the embryo-derived stromal cell lines UG26-1B6 and EL08-1D2 led to an increased production of hematopoietic progenitors in culture. Since, previous studies showed that *Sfrp1* is expressed in bone-lining osteoblast-like cells (Bodine et al., 2004), and the endosteal surfaces of trabecular bones function as a niche in hematopoiesis (Arai and Suda, 2007), we hypothesized that *Sfrp1* might play an important role in the extrinsic regulation of hematopoiesis. Hence, we decided to analyze early hematopoietic cells and the intrinsic and extrinsic contribution of *Sfrp1* deficiency to hematopoietic regulation.

In the present investigation, we found that *Sfrp1* loss leads to increased peripheral blood cell numbers, particularly B220+ and granulopoietic (Gr1+) cells. This observation suggested a dysregulation of hematopoiesis, which is in line with the proposed role of *Sfrp1* as an inhibitor of B lymphopoiesis (Yokota et al., 2008), but which was previously not detected in mice over expressing another Wnt-antagonist, *Dkk1* in osteoblasts (Fleming et al., 2008), or in mice with conditional deletion of *Ctnnb1* (Cobas et al., 2004). However, severe decreases in blood cell numbers were noted in studies in which *Ctnnb1* was stabilized (Kirstetter et al., 2006, Scheller et al., 2006). In the latter mice, it was noted that in the LSK cells, the number of G0/G1 cells

was decreased, suggesting the earliest hematopoietic cells were recruited to enter the cell cycle, but that the number of HSC required to maintain hematopoiesis was, in fact depleted, perhaps because the cells did not return to G0 (Scheller et al., 2006). We clearly show that the general level of Ctnnb1 is decreased in *Sfrp1*^{-/-} LSK, MPP, and CLP, and that P-Ctnnb1 is decreased in LSK cells. Others have shown that *Sfrp1* treatment increases β -catenin levels in Lin⁻ Kit⁺ cells (Yokota et al., 2008). Although these observations suggest that *Sfrp1* may directly inhibit degradation of Ctnnb1 which is normally associated with stimulation of canonical Wnt signalling, these findings would contrast to the general designation of *Sfrp1* as a canonical Wnt inhibitor.

Most likely the balance between canonical and non-canonical signalling dictates the intracellular level of Ctnnb1. An alternative explanation for decreased Ctnnb1 levels with *Sfrp1* loss might be our recent observation that *Sfrp1* binds and inhibits *Wnt5a* (Matsuyama et al., 2009), an interaction which potentially increases the level of Ctnnb1 (Topol et al., 2003). Loss of *Sfrp1* could, therefore increase *Wnt5a*-dependent signalling, thereby indirectly affecting Ctnnb1 stability. A finding in favour of this hypothesis is our observation that a central target of the calcium-dependent non-canonical Wnt pathway, *Pparg*, is increasingly expressed in the absence of *Sfrp1* in MPP. *Pparg* was shown to suppress Ctnnb1 levels in colon cancer (Girnun et al., 2002) and during adipogenesis (Moldes et al., 2003), most probably through a proteasome-dependent mechanism (Sharma et al., 2004). More importantly, *Pparg* is transcriptionally repressed by non-canonical Wnt signalling through the calcium-dependent Camk2-Tak1-Tab2-Nlk in bone marrow mesenchymal progenitors (Takada et al., 2007). These findings suggest that, at least in MPP, in absence of *Sfrp1* the non-canonical calcium-dependent Wnt pathway is up regulated. Considering the complex interplay of Wnt agonists and antagonists within the microenvironment, the identification of the precise mechanism by which *Sfrp1* may be stimulating Ctnnb1 signalling will be difficult and lies beyond the scope of the present paper.

We found that *Ccnd1*, a “classical” transcriptional target of canonical Wnt signalling was decreased in CD34-LSK cells. Hence, our finding that *Sfrp1* deficiency affected cell cycling was not surprising. However, it was unanticipated that the effects of *Sfrp1* on early hematopoietic cells (LSK, MPP) appeared to favour retention in G0/G1,

whereas S-phase entry was increased in more mature B220+ and Gr1+ cells. This suggests that early and mature cells are, in fact, regulated through different *Sfrp1*-modulated signalling pathways. Whereas the decreased cycling activity of LSK and MPP can be attributed by decreased canonical signalling as well as with increased *Pparg* (Altiok et al., 1997), the increase on cycling in mature cells is not explained by this mechanism. Previously, the haploinsufficiency of the non-canonical intermediate *Wnt5a* in adult mice was shown to lead to increased peripheral blood cell numbers, especially B220+ and Gr1+ cells (Liang et al., 2003). This observation shows similarities with our observations in *Sfrp1*^{-/-} mice. Hence the alterations of cell cycling in more mature populations could be explained by deficient non-canonical signalling with little contribution of catenin-dependent canonical signalling.

Our results also imply a possible involvement of *Sfrp1*-dependent signalling events in the regulation of non-Wnt pathways. It was reported that Wnt- and Notch signalling may collaborate to regulate self-renewal of HSC (Duncan et al., 2005). Indeed, our results show that *Sfrp1* deficiency causes an up regulation of the Notch target *Hes1* in both Cd34-LSK and MPP. Since *Hes1* over expression induces quiescence (Yu et al., 2005), this observation suggests collaborating Notch signals could be involved in the observed cell cycle changes seen in early hematopoietic cells. Unexpectedly, we also saw changes in the expression of *Ikbkg*, an NFκB modulator involved in B cell development, and *Runx1*, which is important in hematopoietic specification and lineage maturation, but does not appear to affect HSC behaviour (Ichikawa et al., 2004). The latter was surprising as, though *Runx1* and *Lef1* are both regulated by Groucho family members (Levanon et al., 1998), it was previously unknown that *Runx1* transcription was regulated through the Wnt pathway. We clearly observe a slightly decreased expression of *Runx1* in Cd34-LSK and an increase of *Runx1* in MPP, indicating a tightly developmentally regulated program for *Runx1* expression from Cd34- LSK to MPP cells, which, in part, depends on *Sfrp1*.

Our transplantation experiments revealed that there are no intrinsic defects in HSC caused by *Sfrp1* deficiency. The kinetics of lymphomyeloid lineage engraftment was, in fact, the same in control and *Sfrp1*^{-/-} HSC donors. However, we found a severe loss of maintenance of wild-type HSC in *Sfrp1*^{-/-} recipients, suggesting extrinsic *Sfrp1*-dependence of lymphomyeloid engraftment. This finding is puzzling with regard to its similarity with the results of transgenic mice over expressing *Dkk1* in

osteoblasts (Fleming et al., 2008). In both models, initial engraftment in primary recipients is increased, with a significant drop in engraftment in 2° recipients, suggesting that engrafted HSC are in cycle in both models. Our coculture experiments suggest that progenitors are indeed recruited into cell cycle more on *Sfrp1* knockdown cell lines or *Sfrp1*-deficient primary marrow stromal. Most likely, differences between steady state hematopoiesis and hematopoietic repopulation are, in fact, not comparable. During the regenerative response after myeloablation, the microenvironment undergoes significant remodelling (Slayton et al., 2007). Also, there is a rapid up regulation of *Wnt10b* in both stromal and hematopoietic cells, with a concomitant stabilization of *Ctnnb1* and up regulation of *Myc* and *Axin2* (Congdon et al., 2008). Since down regulation of canonical signalling is what the *Dkk1* transgenic and *Sfrp1* deficient models have in common, it appears that canonical Wnt signalling is the main driving force in hematopoietic regeneration after myeloablative insult.

In conclusion, our study shows the value of studying stromal cell lines to uncover factors involved in extrinsic regulation of HSC. The present study demonstrates that microenvironmental *Sfrp1* affects both catenin-dependent canonical and *Pparg*-dependent non-canonical Wnt signalling to regulate cell cycling and gene expression in HSC and lineage-committed MPP. Our observations show, that *Sfrp1* does not have major intrinsic roles in lymphomyeloid engraftment and that loss of *Sfrp1* severely impairs HSC maintenance in an extrinsically regulated manner.

5.5 Experimental procedures

Mice

Sfrp1^{-/-} mice were bred on a 129xC57BL/6.J (129B6) background (Satoh et al., 2006). Age and sex matched 129B6, C57BL/6.Pep3b.Ptprc (Ly5.1), and (129xLy5.1)F1 (129Ly5.1) mice were used as controls in all experiments. Mice were kept in Microisolators under SPF conditions according to FELASA recommendations.

Cell lines

The embryo-derived stromal cell lines EL08-1D2, UG26-1B6, UG15-1B7, AM20-1B4, EL28-1B3, and AM30-3F4 were cultured as described previously (Oostendorp et al., 2002). Stable knockdown cells for *Sfrp1* were made using lentiviral shRNAmir (Open Biosystems, Huntsville, USA) followed by puromycin-selection. Puromycin was removed from the medium 3 days prior to the experiments.

Tissue samples

Blood samples were taken retro-orbitally with 0,8mm capillaries (Neolab, Heidelberg, Germany) and collected in 1.2 ml heparinised tubes (Sarstedt AG, Nümbrecht, Germany). Blood cells were counted on a Scil Vet ABC (Scil Animal Care, Viernheim, Germany). Bone marrow cells were flushed from femurs and tibias with HF2+ buffer (Hank's balanced salt solution, supplemented with 2% FCS, 10 mM HEPES buffer and antibiotics). Spleen and thymus were passed through 70µm nylon Cell Strainer (BD Biosciences, Erembodegem, Belgium). WBC were counted after erylisis with ammonium chloride solution (Stem cell technologies, Vancouver, Canada). Viable cell were counted using Trypan Blue (Invitrogen) in a Neubauer hemocytometer.

FACS analysis

Cell suspensions were stained with antibodies in HF2+ buffer for 15 minutes on ice in the dark. Hematopoietic populations were separated with the following antibodies: biotinylated Gr-1, phycoerythrin (PE)-labelled Cd4, Cd8, and Cd117 (Kit), PE-Cy5-labelled B220, fluorescein isothiocyanate (FITC)-labelled Cd34 and Cd48, Pacific Blue-labelled Cd16/32 (FcγR), Alyophycocyanin (APC)-labelled Cd117 and IL7R, and PE-Cy7-labelled anti Ly-6A/E (Sca1) were all obtained from eBiosciences (San Diego, CA, USA) and PE-labelled Cd150 from BioLegend (San Diego, CA, USA). Lineage stains were performed with biotinylated lineage cocktail (Miltenyi Biotec, Bergisch Gladbach, Germany), supplemented with biotin-labelled CD3 and IL-7R antibodies (eBiosciences). Blood samples were analysed with following antibodies: biotinylated Gr-1 (eBioscience) labelled with PECy5 streptavidin (Caltag), Cd4 – PECy5 (BD Pharmingen), Cd8a – PECy5 (eBioscience) or B220 – PECy5 (BD Pharmingen). In transplantation experiments Cd45.2 – FITC (BD), Cd45.1 – PE (BD Pharmingen) antibodies were used additionally.

FACS analyses were performed on a Coulter EPICS XL (Beckman Coulter GMBH, Krefeld, Germany) or CyAn ADP Lx P8 (Beckman Coulter) flow cytometers. FACS data were analysed using FlowJo 8.8.3 software (Tree Star, inc. Ashland, USA).

Cell populations were sorted on MoFlo (Cytomation-Beckman Coulter) supplied with Summit 4.3 software (Beckman Coulter).

Hematopoietic colony assays

For colony forming assay BM cells (2.5×10^4), or cocultures initiated with 1×10^4 of Lin-cells were cultivated in methylcellulose (MethoCult[®] GF M3434, Stemcell technologies, Vancouver, Canada) on 3,5 mm dishes for ten days at 37 °C, 5 % CO₂. Colonies formed were counted under a microscope.

The stromal cells used for cocultures were irradiated with 30Gy (UG26-1B6) and 15Gy (primary stroma) by a Mevatron KD2 (Siemens, Munich, Germany).

In the rescue experiments, half of the medium was replaced on each third day with conditioned medium (CM), harvested and 0.22 µm filtered from confluent grown *Sfrp1*^{+/+} primary BM stroma.

Human recombinant SFRP1, (R&D Systems, Minneapolis, USA) was added at a concentration of 1µg/ml when used to evaluate its effect.

Cell cycle analysis

For analysis of cell cycle animals were intraperitoneally injected with 1 mg of BrdU. After 3 hours BM cells were flushed and stained with surface markers prior to BrdU detection with BrdU labelling kit as described by the manufacturer (BD Biosciences).

Transplantation assay

The Cd45 congenic system (Cd45.1 and Cd45.2) was used to distinguish donor from recipient cells. 10 to 12 weeks old lethally irradiated recipient mice received donor cells via the tail vein on the same day. In primary transplantations (1°) 2×10^5 BM cells were used and in secondary transplants (2°) - 1×10^6 .

For the limiting dilution assay, 1.5×10^5 , 5×10^4 or 2×10^4 BM cells from WT or *Sfrp1*^{-/-} mice were injected with 2×10^5 (129Ly5.1) competitor cells into WT recipients. Mice

with engraftment over 1% of myeloid and 1% lymphoid lineages are defined as positive mice.

Transplanted mice received 1 mg/ml neomycin sulphate (Sigma) and 500 units/ml Polymyxin B (Sigma) in the drinking water. At week 16 weeks post transplantation mice were sacrificed, BM -, spleen-, and blood cells were analysed.

Intracellular stains

For intracellular staining three million of BM cells were stained for surface markers prior to fixation and permeabilization with BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences). In brief, cells were fixed and permeabilized in Cytofix/Cytoperm buffer for 20-25 minutes at room temperature in the dark. After fixation and permeabilization, cell were washed with Perm/Wash buffer and incubated with primary antibody diluted in the staining buffer over night at 4°C. Antibodies used: rabbit anti Ctnnb1, rabbit anti Ser33/Thr41 P-Ctnnb1 (both from Cell Signalling Technology, Boston, USA), rabbit anti Jnk/Mapk8 (Santa Cruz Biotechnology, Heidelberg, Germany), control preimmune rabbit immunoglobulin (Jackson ImmunoResearch, Newmarket, Suffolk, UK), followed by FITC-labelled anti rabbit antibodies (Jackson ImmunoResearch). Stained cells were analysed on CyAn ADP Lx P8 (Beckman Coulter) flow cytometer.

Immunocytofluorescence staining

For the single cell staining assay 200 sorted LSK, MPP and CLP were spotted on poly-L-lysine-coated glass slides. After a short incubation on ice cells were fixed with 4% PFA. Fixed cells were incubated in 10% FCS, 0,1% Triton-X in PBS at room temperature for 1 hour and stained overnight with anti- Ctnnb1, L54E2 Alexa Fluor 488-conjugated Mouse antibody (Cell Signalling). The cell nuclei were counterstained with DAPI (4,6-diamino-2-phenylindole, dihydrochloride) (Invitrogen) and mounted in Prolong® Gold Antifade Reagent. Staining was assessed using a 100X magnification on a Leica DM RBE fluorescent microscope (Leica, Wetzlar, Germany). The fluorescence intensity of each individual cell was quantified with ImageJ (NIH, Bethesda, USA) software. The nuclear expression was quantified in the area corresponding to the DAPI positive area. The cytoplasmic expression was quantified in the cell compartment after subtraction of the “nuclear” area.

Gene expression analysis

mRNA was isolated from sorted cells by using the Dynabeads® mRNA DIRECT™ Micro Kit (Invitrogen) as described by the manufacturer. cDNA was amplified using Omniscript® RT kit (Qiagen GmbH, Hilden, Germany) as described by manufacturer. The following Quantitative PCRs were performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster city, USA) according to manufacturer instructions, and analyzed on a Applied Biosystems 7900HT (Applied biosystems). Gene expression is presented as a relative value ($2^{\Delta Ct} \times 100\%$) compared to the expression levels of housekeeping genes: *Rpl13a*, *Rps21*, *Hprt1*, and *Ytdhf1*. Primer sequences are shown in table S1.

Statistics

Unpaired students T-test was used for the statistical analyses using the InStat statistical package (GraphPad Software, Inc., La Jolla, USA). The limiting dilution experiment was evaluated using the L-Calc software package (Stem Cell Technologies).

5.6 Acknowledgements

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5.7 Supplementary data: Renström et al. 2009.

TABLE S1

Gene	RefSeq	Forward	Reverse
<i>Ccnd1</i>	NM_007631.2	GCGTACCCTGACACCAATCT	CACAACCTTCTCGGCAGTCAA
<i>Dkk1</i>	NM_010051	ATATCACACCAAAGGACAAGAAGG	AGGTTTACAGATCTTGGACCAGAA
<i>Fzd4</i>	NM_008055.4	TGACAACCTTTCACGCCGCTC	TACAAGCCAGCATCGTAGCCACAC
<i>Hes1</i>	D16464.1	CTGTGGGAAAGAAAGTTTGGGAAG	GCTCCAGATCCTGTGTGATCC
<i>Hprt1</i>	NM_013556.2	GCTTGCTGGTGAAAAGGACCTCTCGAAG	CCCTGAAGTACTCATTATAGTCAAGGGCAT
<i>Ikbkg</i>	NM_010547	GACAAGGCCTCTGTGAAAGC	GCTGTTGCCTCAGATCTTCC
<i>Lef1</i>	NM_010703	CAGCCTGTTTATCCCATCAC	TCCTGTTTGACCTGAGGTGT
<i>Lmo2</i>	NM_008505.3	ATTGCCTCAGCTGTGACCTC	CCCATTGATCTTGGTCCACT
<i>Pparg</i>	NM_001127330.1	CTGTGAGACCAACAGCCTGA	AATGCGAGTGGTCTTCCATC
<i>Rpl13a</i>	NM_009438.4	CCCTCCACCCTATGACAAGA	TTCTCCTCCAGAGTGGCTGT
<i>Rps21</i>	NM_025587.2	CTGGACAAGTGAGCAGGGA	GCCTGTGGTCCTATCAACCT
<i>Runx1</i>	NM_001111021.1	TACCTGGGATCCATCACCTC	AGGCGCCGTAGTATAGATGG
<i>Ythdf1</i>	NM_173761.3	TTGGTCCAGCCACAGTATCA	CAGGACAGGGTGGGATTCTA

Table S1. Primer sequences used for real-time PCR. Shown are all primer pairs used for gene expression studies. Primers used for genotyping of *Sfrp1*^{-/-} mice were described previously (Satoh et al., 2006).

TABLE S2

<i>1° recipient</i>	<i>Number of BM cells harvested (per four long bones)</i>	<i>Number of cells transplanted into 2° recipients</i>	<i>Positive* / Total (in PB) in 2° recipients</i>	<i>HSC Frequency in 1° BM</i>	<i>Range (95% confidence interval)</i>	<i>Calculated number of HSC in 1° BM</i>
<i>Control</i>	7.0×10^7	1×10^6	8/9	1 in 455,000	1 in 296,000 to 1 in 699,000	154
<i>Sfrp1^{-/-}</i>	5.9×10^7	1×10^6	10/13	1 in 682,000	1 in 963,000 to 1 in 483,000	87

Table S2. Estimated HSC frequencies in BM of 1° control or *Sfrp1^{-/-}* recipients.

Sixteen weeks after transplantation of 2×10^5 BM WT cells in lethally irradiated control or *Sfrp1^{-/-}* mice, 1×10^6 BM cells were serially transplanted into control 2° recipient mice. Sixteen weeks later, donor engraftment was determined. Mice were indicated “positive” when more than 1% of the peripheral blood cells belonged to the myeloid lineage (Cd11b+ or Gr1+) and more than 1% lymphoid (Cd4, Cd8a or B220+ cells) were detected. Since only one single dose of cells was transplanted, the HSC frequency is an estimate, which was calculated using the L-Calc software package (Stem Cell Technologies). The number of HSC per four long bones was calculated by dividing the number of cells harvested from the primary recipient mice by the estimated HSC frequency.

FIGURE S1

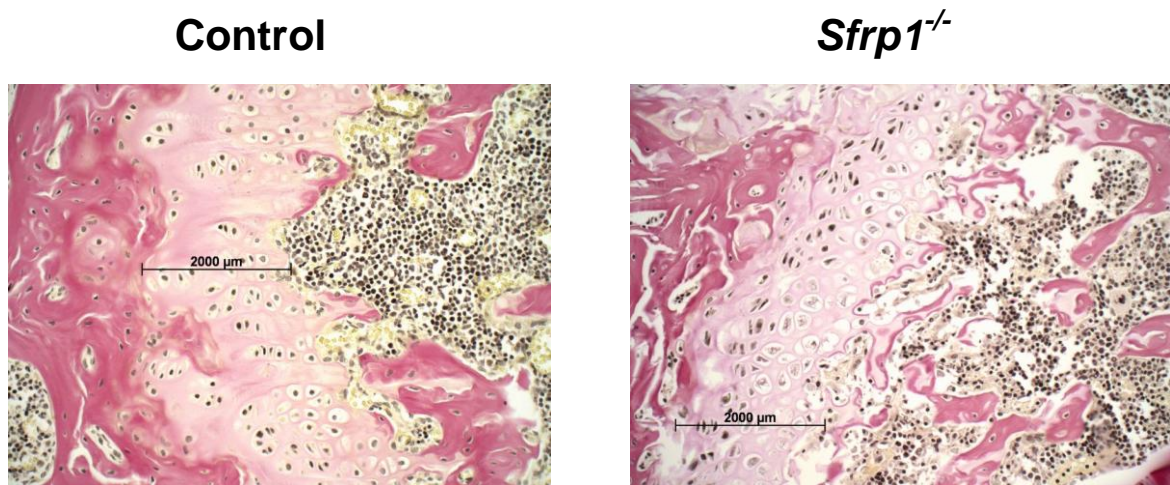


Figure S1. Growth plates of 8-10 week-old *Sfrp1*^{-/-} mice are similar.

Paraffin section of femurs from 10 week old control and *Sfrp1*^{-/-} mice with Van Gieson staining (original magnification 20x). The growth plate size is 2000 µm in both control and *Sfrp1*^{-/-} mutant mice. Chondrocytes seemed slightly disorganized and their numbers tended to be slightly higher in *Sfrp1*^{-/-} mice.

FIGURE S2

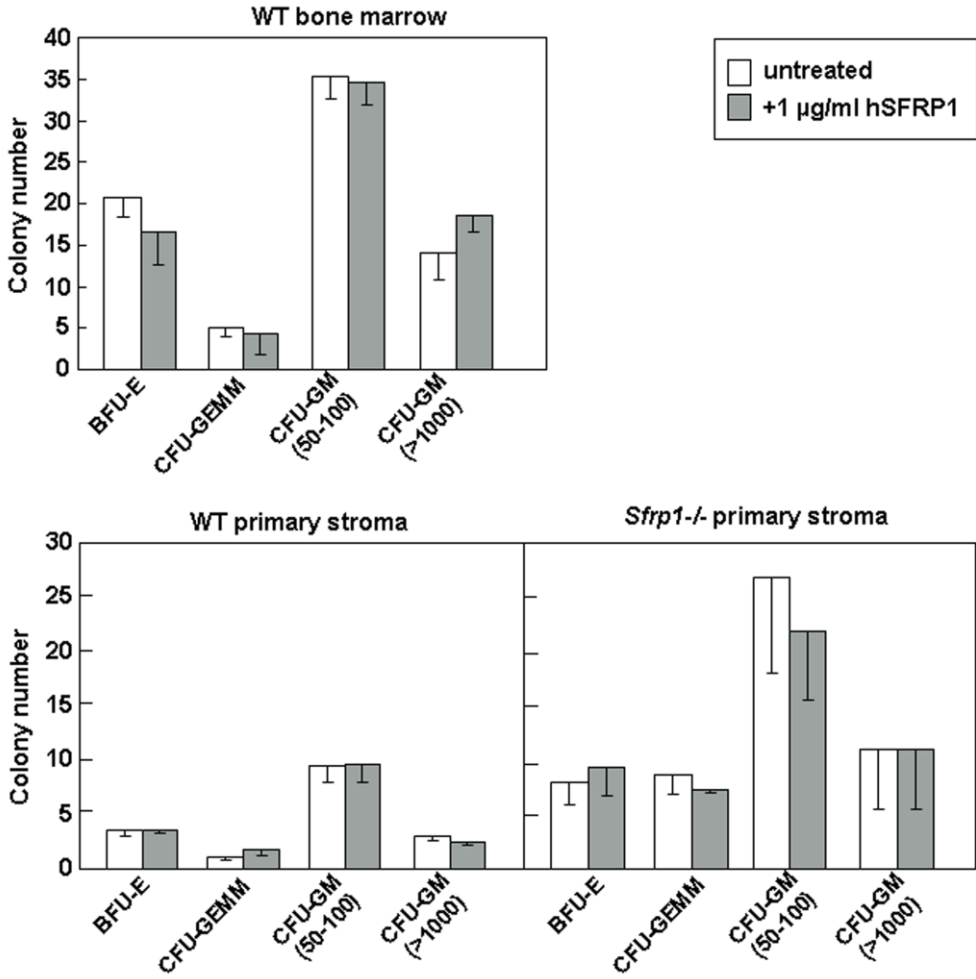


Figure S2. No effect of recombinant *Sfrp1* on hematopoietic colony formation

a) Colonies formed from 25000 of BM cells from control mice with and without addition of recombinant human *Sfrp1* (1 µg/ml [≈30 nM]) to the semi-solid methylcellulose culture medium. **b)** Colonies formed from 10000 of wt Lin⁻ cells cocultured for 2 weeks on primary stroma with and without addition of recombinant human *Sfrp1* (1 µg/ml [≈30 nM]) to stroma medium every third day .

FIGURE S3

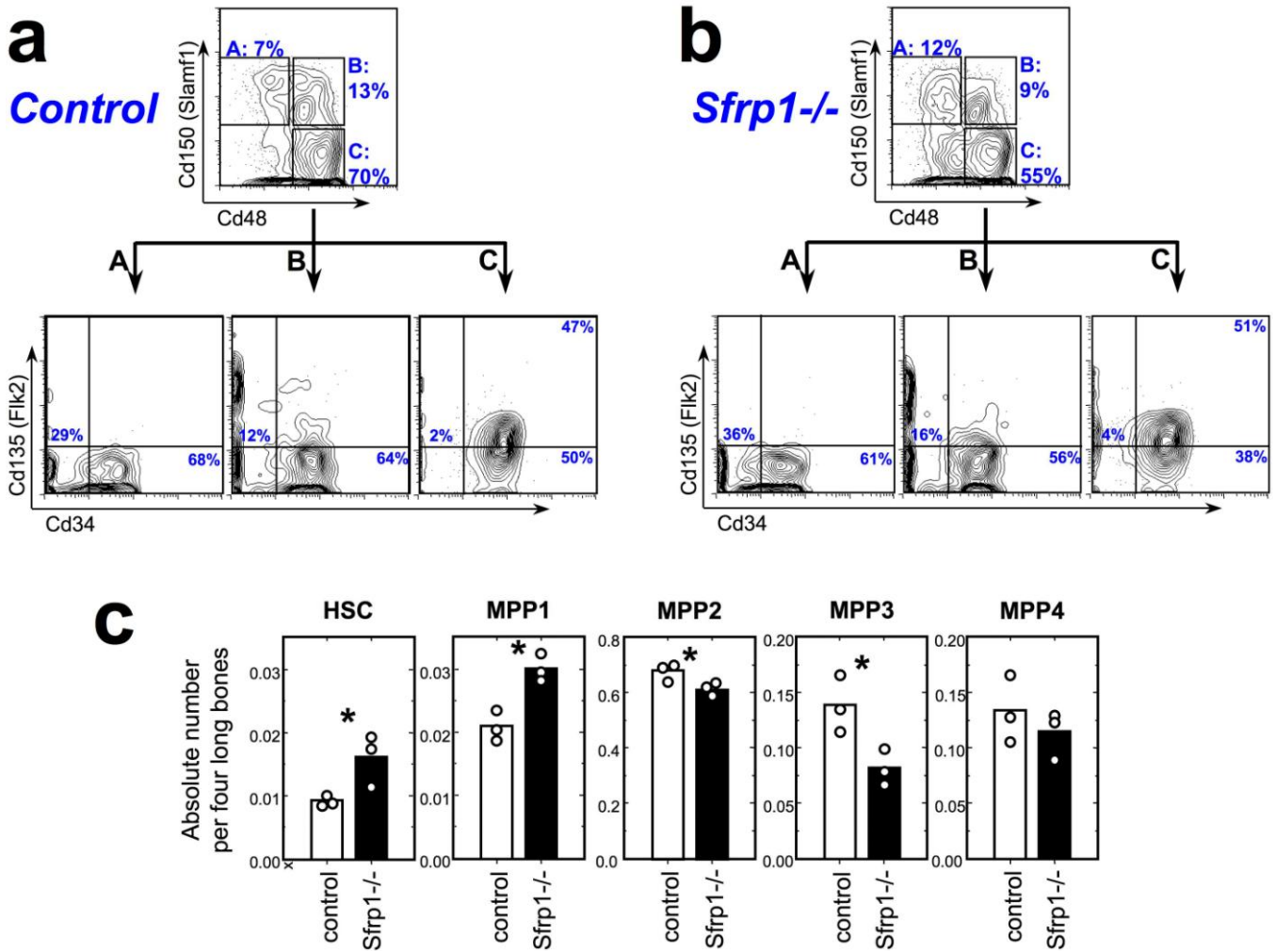


Figure S3. Expression of SLAM family markers CD150 (Slamf1) and CD48 (Slamf2) on LSK cells from control or *Sfrp1*^{-/-} LSK cells.

BM cells from control (a) or *Sfrp1*^{-/-} (b) mice were stained for Lin, Kit, Ly-6A/E (Sca1), Cd34, Cd48, Cd150 and Cd135 (Flk2). The gating strategy was similar as reported by others (Wilson et al., 2008). In the latter report, within the LSK cell fraction, five cell types with decreasing self-renewal ability and increasing cell cycling activity were defined:

Population	Gate	Cd34	Cd135 (Flk2)	Cd150 (Slamf1)	Cd48 (Slamf2)
HSC	A	-	-	+	-
MPP1	A	+	-	+	-
MPP2	B	+	-	+	+
MPP3	C	+	-	-	+
MPP4	C	+	+	-	+

We quantitated the number of each of these fractions as total number of cells per four long bones ($\times 10^6$) in (c). A total of three animals per group were analysed. Comparisons between control and *Sfrp1*^{-/-} animals were performed using the unpaired student's t-test. P-value: *: $p < 0.05$.

FIGURE S4

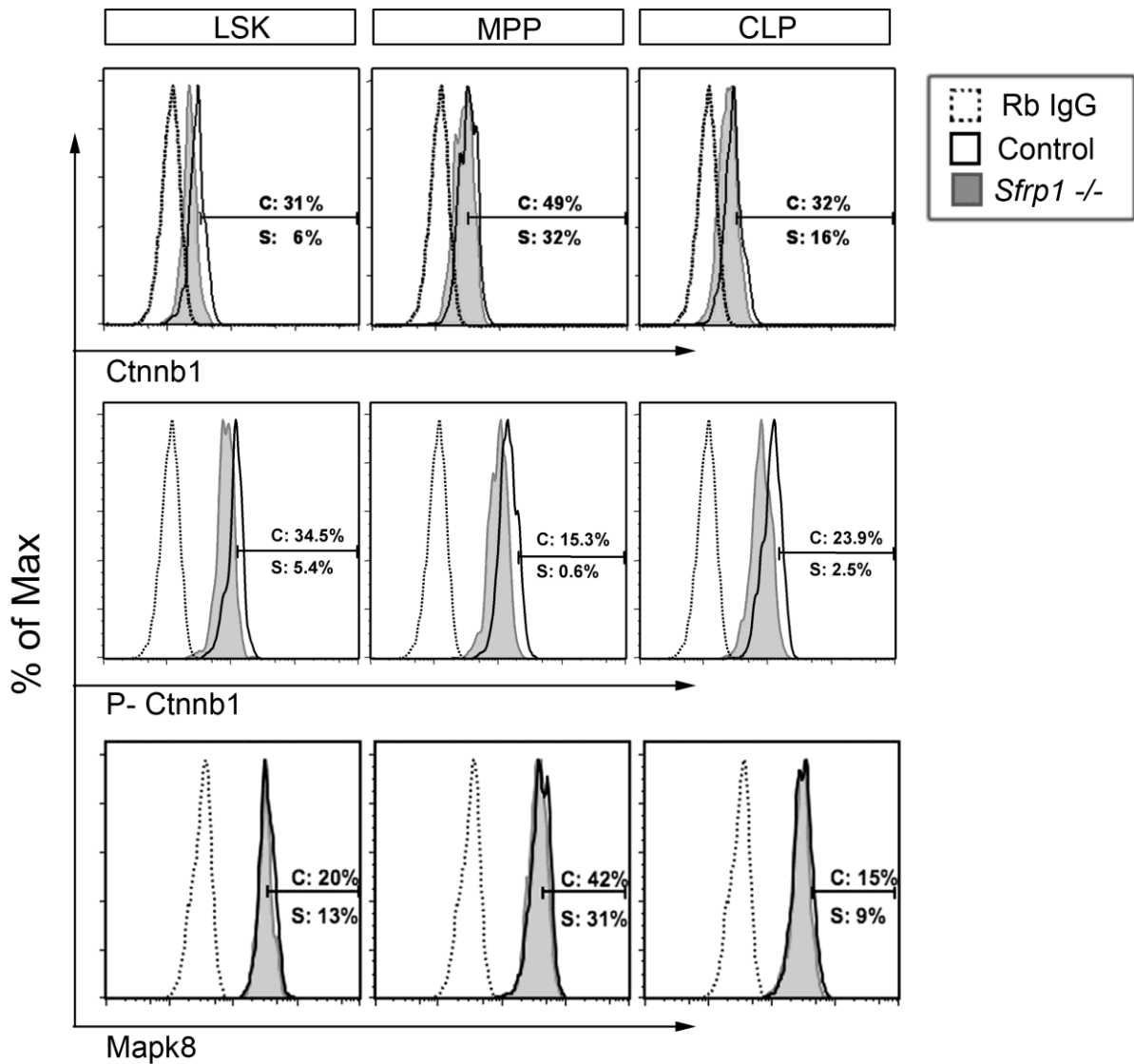


Figure S4. Gating strategy for Ctnnb1, P-Ctnnb1 and Mapk8 high positive cells

Shown is a histogram of Ctnnb1, P-Ctnnb1 and Mapk8 positive cells in LSK, MPP and CLP. The percentages of highly positive control (C) and *Sfrp1*^{-/-} (S) cells are given within the gates. Dashed line represents cells positive for rabbit IgG, used as a secondary antibody and is gated on Lin⁻ cells. Black lines correspond to the control and filled profiles correspond to the *Sfrp1*^{-/-} mice.

FIGURE S5

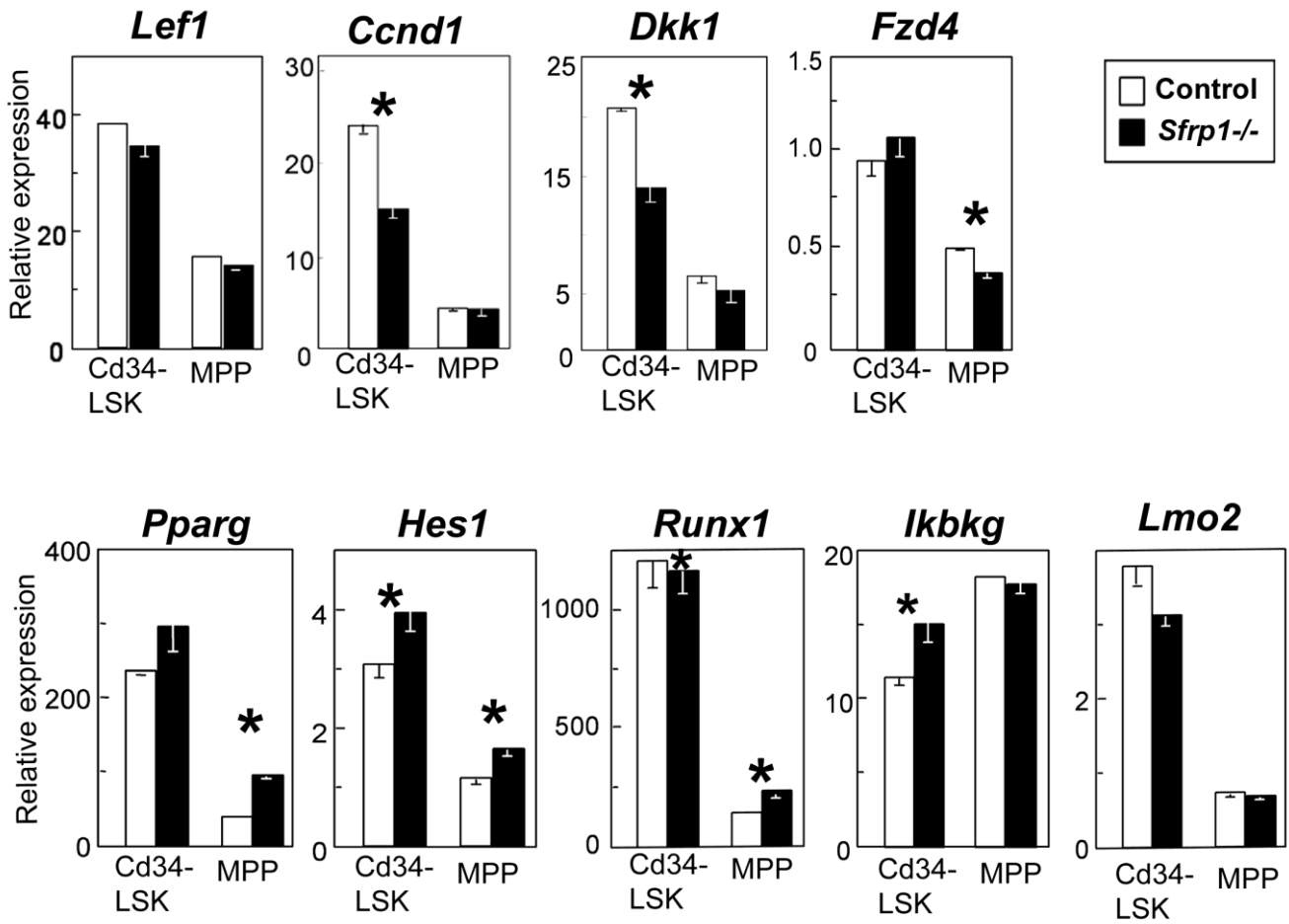


Figure S4. Expression levels of different hematopoietic regulators in Cd34-LSK and MPP of *Sfrp1*^{-/-} mice.

Show is the non-normalized relative expression ($2^{\Delta Ct}$ value) in LSK- and MPP cells of the genes investigated in this study. All values are shown as mean \pm SEM. Statistical comparisons were performed using the unpaired student's t-test. P-value: *: p < 0,05.

FIGURE S6

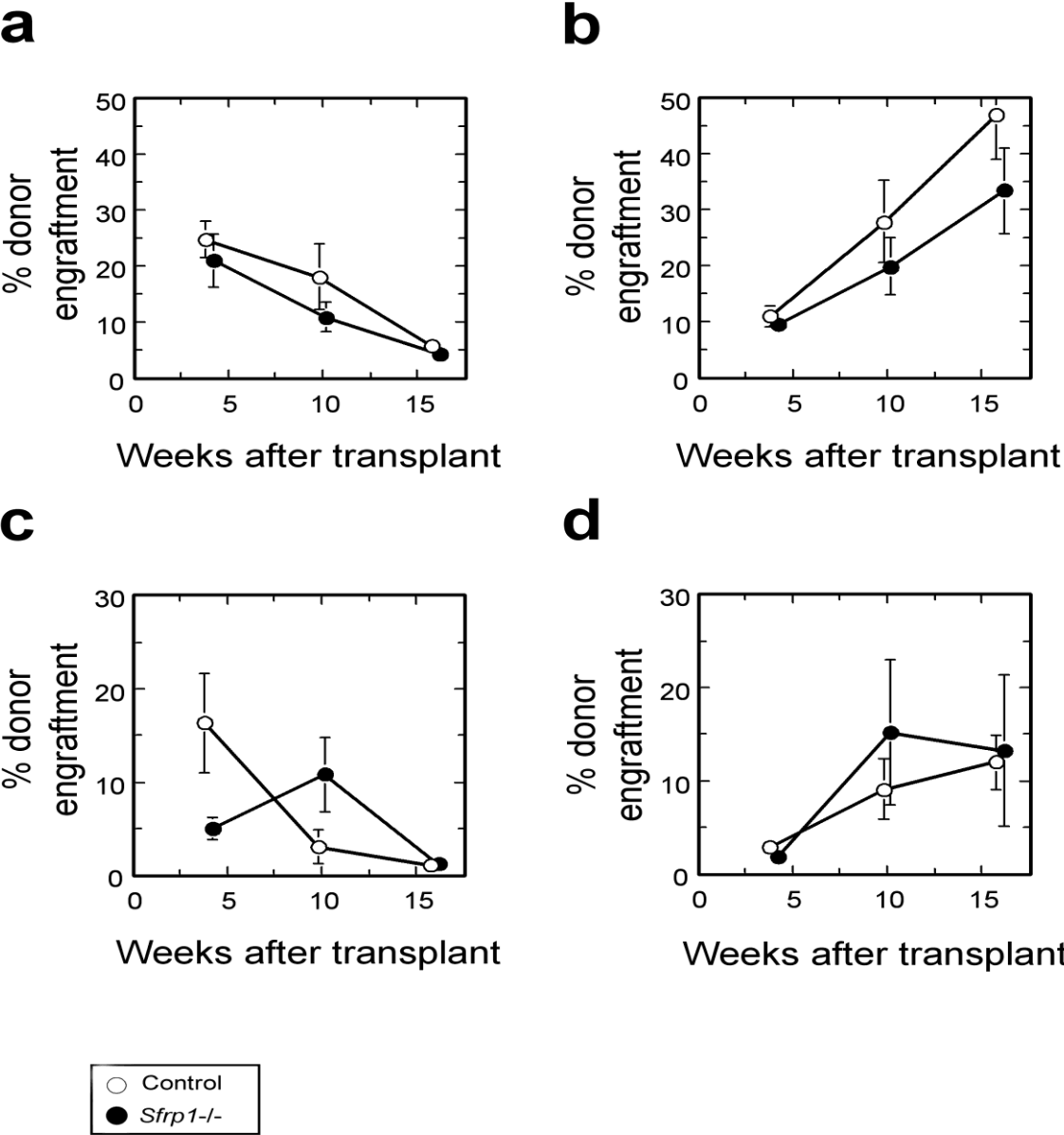


Figure S6. Myeloid and lymphoid engraftment pattern at two different donor cell doses after transplantation, over time.

Shown is the level of engraftment of myeloid cells (a, c) and lymphoid cells (b, d), in wild-type recipient mice transplanted with wild-type (open symbols) or *Sfrp1*^{-/-} (closed symbols) donor bone marrow. The top row (a, b), represents mice transplanted with 1.5x10⁵ bone marrow cells (n=5, each group), the bottom row represents mice transplanted with 2x10⁴ bone marrow donor cells (n=5 (control), and n=6 (*Sfrp1*^{-/-}) donors). All values are shown as mean ± SEM.

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Chapter 6: Characterization of the mature hematopoietic cell populations in *Sfrp1*^{-/-} mice.

6.1 Introduction

During the studies of the *Sfrp1* deficient mice we also did more comprehensive analyses of the more commonly investigated hematopoietic cell populations in several hematopoietic tissues such as blood, bone marrow, thymus, spleen and lymph nodes. These findings were omitted in the article published in *Cell stem cells* due to practical and scientific reasons.

The hematopoietic system is made up by many different types of cell which can be expressing different markers depending on in which tissue they are found despite their common background. T cells for example are most commonly investigated in the thymus, which is contains almost exclusively T cells, even though there are T cells to be found in other tissues as well but they are not as numerous or have the same characteristics. A similar thing can be said about B cells, even though not to the same degree as with the T cells, which are more common in the spleen than elsewhere but they also occur in reasonable amounts in blood and bone marrow, albeit with different characteristics. This makes the detailed investigation of the different hematopoietic tissues a necessity. A number of papers have been published with detailed description of the different mature cell populations and which antibodies to be used, the ones used in this study was (Otero and Rickert, 2003) for B cells, (Zhang et al., 2003), for erythroid cells and (Bronte et al., 2000) for myeloid cells. Using the characterizations used in the previously mentioned papers it was possible to get a good overview of the *Sfrp1*^{-/-} hematopoiesis. The findings suggest that there is a disturbance in more mature hematopoiesis with alterations primarily in early and late B cell populations but also in myelopoiesis, represented by shifted erythropoiesis and immature myeloid progenitors. Interestingly though, even when all other hematopoietic populations seem to be altered the T lymphopoiesis in the thymus remained unchanged.

6.2 Results

By using the definitions used by Otero et al., 2003, it was possible to stain and distinguish several different B cell sub-populations; the ones analyzed here were the overall B220 positive cells and out of these we further analyzed pre-B cells that were double negative for IgM and Cd43, pro-B cells that are Cd43 positive but still IgM negative and the more mature B cells that are IgM positive and Cd43 negative. B cells in *Sfrp1*^{-/-} mice, in blood, bone marrow and spleen, display a disturbed maturation with a loss of pro B cells (B220+IgM-Cd43+) and an accumulation of more mature ones (B220++IgM+), Figure 19 and Tables 6, 7 and 8.

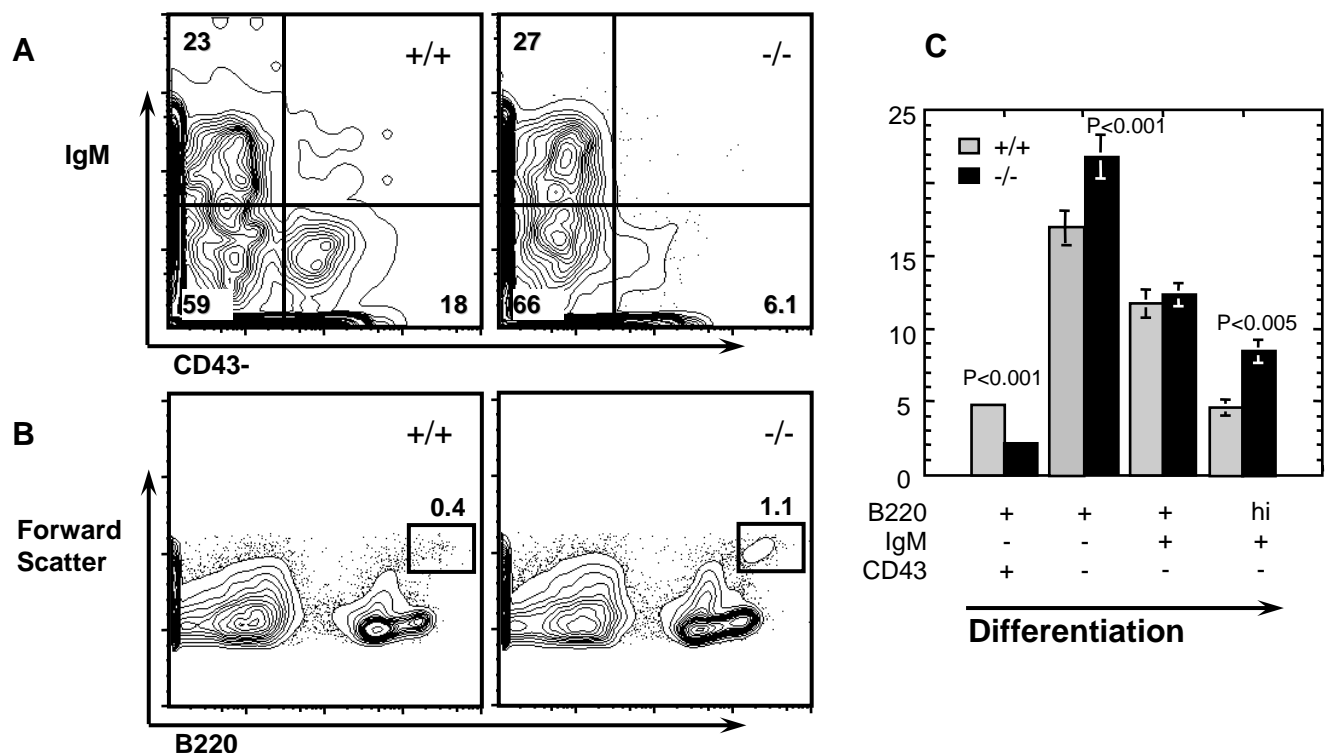


Figure 19. B cell sub-population gating strategy. **A)** Showing B220+ cells here gated for IgM and Cd43 expression. **B)** Gating for B220 high, larger cells. **C)** Diagram showing the different B cell populations and how they compare between *Sfrp1*^{+/+} and *Sfrp1*^{-/-} mice.

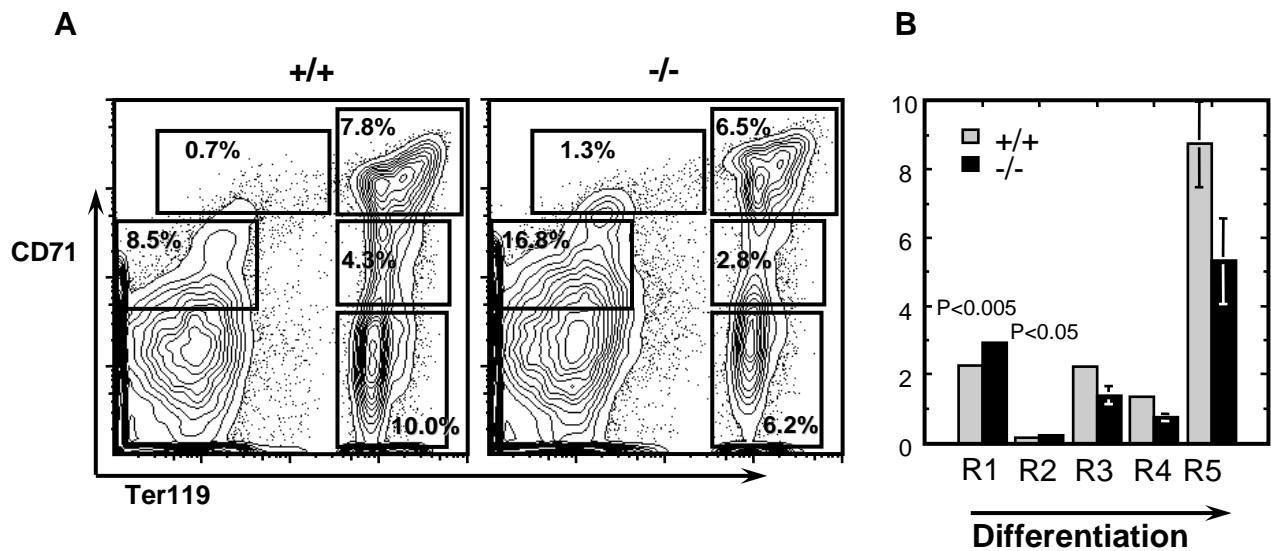


Figure 20. Erythroid differentiation. **A)** Showing the R1-R5 classification using Cd71 and Ter119 on whole bone marrow cells. **B)** Diagram showing the differences in erythrocyte differentiation between *Sfrp1*^{-/-} and WT mice.

Erythrocytes mature in the bone marrow and can be categorized by a system established by Zhang and co-workers which divides the differentiation process into five different stages called R1-R5. We can now show that erythrocyte maturation is disturbed by the loss of *Sfrp1* resulting in an increase of early stage erythrocytes, Cd71⁺ Ter119⁻ (R1) and Cd71⁺ Ter119^{low} (R2), and a slight but not statistical significant reduction of mature erythrocytes Cd71⁻ Ter119⁺ (R5), Figure 20, and Table 6.

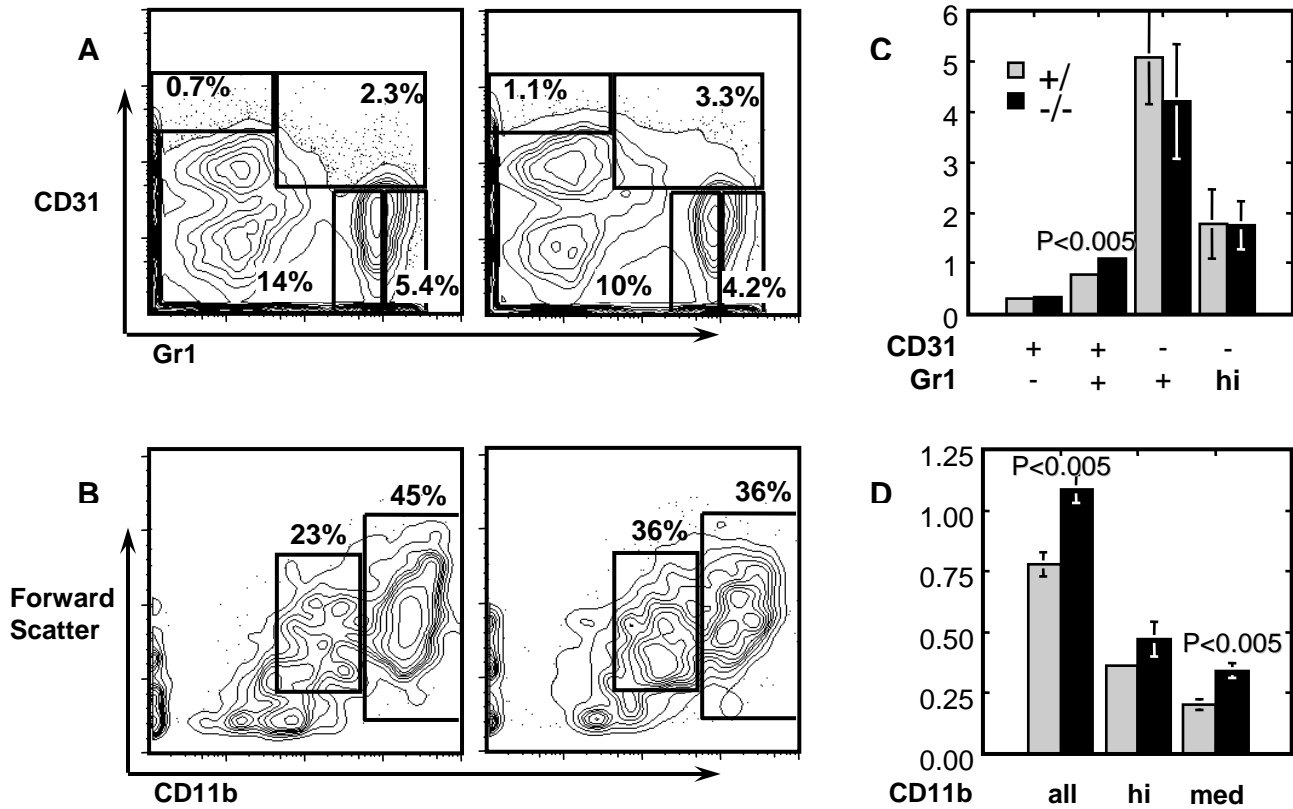


Figure 21. Myeloid differentiation. **A)** Gating strategy for myeloid cells using Cd31 and Gr1. **B)** Cd31+Gr1+ cells gated for Cd11b to reveal the immature myeloid progenitor cells which are Cd11b_{med}. **C)** Diagram comparing Cd31 and gr1 expression in *Sfrp1*^{-/-} mice and WT mice. **D)** Immature myeloid progenitor diagram comparing *Sfrp1*^{+/+} and *Sfrp1*^{-/-}.

Myelopoiesis is altered by the absence of *Sfrp1*, especially the Cd31+Gr1+ cells, which are elevated, and the increased fraction of immature myeloid progenitors (Cd31+Gr1+Cd11b+), described by Bronte and co-workers (Bronte et al., 2000), Figure 21 and Tables 6, 7 and 8.

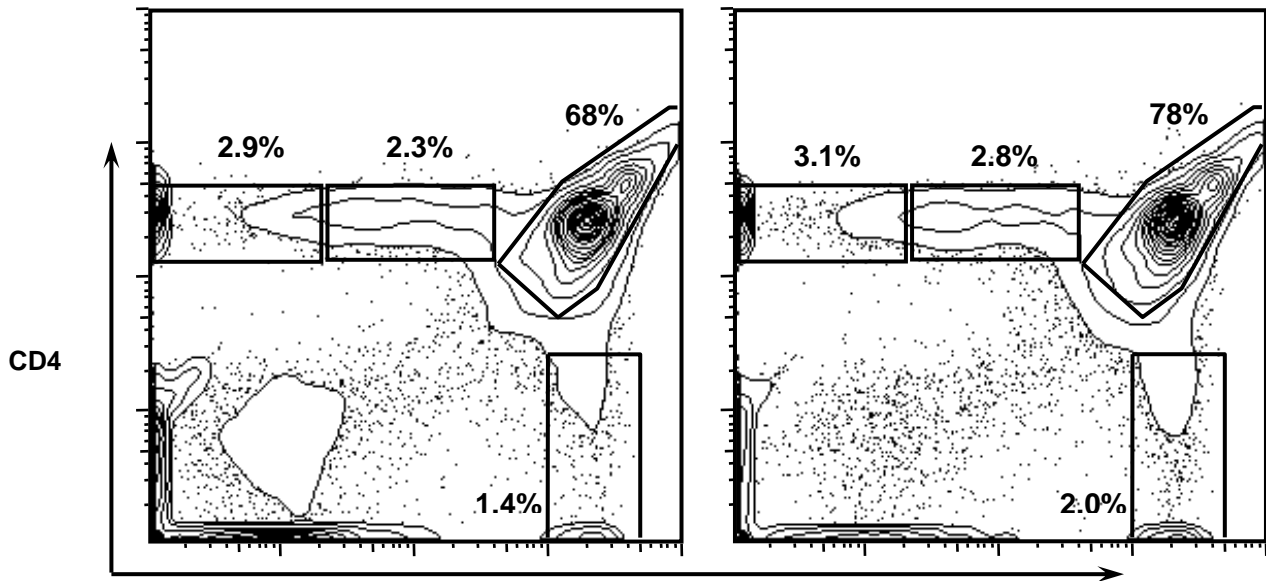


Figure 22. T-lymphopoiesis in the thymus. Gating strategy for determining T cell population distribution in the thymus of *Sfrp1*^{-/-} and WT animals.

However, T-lymphopoiesis, gating according to (Moore and Zlotnik, 1995), remains statistically unchanged in mice lacking *Sfrp1*, Figure 22 and Tables 6, 7, 8 and 9.

This was surprising since all other major hematopoietic cell types exhibited differences in some way.

Bone marrow	P-value	Mean <i>Sfrp1</i> ^{-/-}
Myeloid cells		
CD31+	<0,001	1,38
Gr1 low CD11b+	0,170	1,19
Gr1+ Cd11b+	0,210	1,14
Gr1+ CD31+	0,007	1,75
T and NK cells		
CD4+	0,105	1,60
CD4+ CD8a+	0,349	1,21
CD8a+	0,457	1,28
Nk1.1+	0,431	0,91
B cells		
B220+	0,001	1,38
CD43+ IgM- B220+	<0,001	0,53
CD43- IgM- B220+	0,094	1,14
IgM+ CD43+ B220+	0,660	1,11
IgM+ CD43 - B220+	0,927	1,01
Erythroid cells		
R1	<0,001	1,66
R2	<0,001	1,75
R3	0,447	0,92
R4	0,307	0,88
R5	0,035	0,69

Table 6. The relative representation of hematopoietic sub-populations in mouse bone marrow. Shown as significance value (P-value) and the mean normalized value comparing *Sfrp1*^{-/-} and WT mice, with 1 representing WT. Values <0,05 are considered significant.

Spleen	P-value	Mean <i>Sfrp1</i>^{-/-}
Myeloid cells		
Cd31+	0,519	0,95
Gr1 low CD11b+	0,433	0,90
Gr1+ Cd11b+	0,128	1,97
Gr1+ CD31+	0,749	1,06
T and NK cells		
Cd4+	0,808	0,98
Cd4+ Cd8a+	0,004	0,70
Cd8a+	0,160	0,85
Nk1.1+	<0,001	0,71
B cells		
B220+	0,068	0,88
Cd43+ IgM- B220+	0,002	0,76
Cd43- IgM- B220+	0,384	0,92
IgM+ CD43+ B220+	0,155	0,83
IgM+ CD43 - B220+	0,118	1,03
Weight	0,083	0,85

Table 7. The relative representation of hematopoietic sub-populations and weight of the spleen. Shown as significance value (P-value) and the mean normalized value comparing *Sfrp1*^{-/-} and WT mice, with 1 representing WT. Values <0,05 are considered significant

Blood	P-value	Mean <i>Sfrp1</i>^{-/-}
Myeloid cells		
CD31+	0,100	0,93
Gr1 low CD11b+	0,018	2,64
Gr1+ Cd11b+	0,122	5,51
T and NK cells		
CD4+	0,055	0,75
CD4+ CD8a+	0,184	0,34
CD8a+	0,002	0,50
NK1.1+	0,041	0,64
B cells		
B220+	<0,001	2,09
CD43+ IgM- B220+	0,001	0,41
CD43- IgM- B220+	0,042	1,16
IgM+ CD43+ B220+	0,049	0,40
IgM+ CD43 - B220+	0,005	1,21

Table 8. The relative representation of hematopoietic sub-populations in the blood.

Shown as significance value (P-value) and the mean normalized value comparing *Sfrp1*^{-/-} and WT mice, with 1 representing WT. Values <0,05 are considered significant.

Thymus	P-value	Mean <i>Sfrp1</i> ^{-/-}
T cells		
CD4 CD8a low	0,727	0,96
CD4+	0,442	0,93
CD8a+	0,786	1,03
DN	0,993	1,00
DP1	0,970	1,00
DP2	0,964	0,99

Table 9. The relative representation of T cell sub-populations of the thymus. Shown as significance value (P-value) and the mean normalized value comparing *Sfrp1*^{-/-} and WT mice, with 1 representing WT. Values <0,05 are considered significant.

6.3 Materials and methods

Mice

Sfrp1^{-/-} mice were bred on a 129xC57BL/6.J (129B6) background (Sato et al., 2006). Age and sex matched 129B6, C57BL/6.Pep3b.Ptprc (Ly5.1), and (129xLy5.1)F1 (129Ly5.1) mice were used as controls in all experiments. Mice were kept in Microisolators under SPF conditions according to FELASA recommendations.

Tissue samples

Blood samples were taken retro-orbitally with 0,8mm capillaries (Neolab, Heidelberg, Germany) and collected in 1.2 ml heparinised tubes (Sarstedt AG, Nümbrecht, Germany). Blood cells were counted on a Scil Vet ABC (Scil Animal Care, Viernheim, Germany). Bone marrow cells were flushed from femurs and tibias with HF2+ buffer (Hank's balanced salt solution, supplemented with 2% FCS, 10 mM HEPES buffer and antibiotics). Spleen and thymus were passed through 70µm nylon Cell Strainer (BD Biosciences, Erembodegem, Belgium). WBCs were counted after erylisis with ammonium chloride solution (Stem cell technologies, Vancouver, Canada). Viable cell were counted using Trypan Blue (Invitrogen) in a Neubauer hemocytometer.

FACS analysis

Cell suspensions were stained with antibodies in HF2+ buffer for 15 minutes on ice in the dark. Hematopoietic populations were separated with the following antibodies: biotinylated Gr-1 and Cd71, phycoerythrin (PE)-labelled Cd4, Ter119 and IgM, PE-

Cy5-labelled Cd11b, B220 and Cd8a, fluorescein isothiocyanate (FITC)-labelled Cd43, Cd31 and Nk1.1 were all obtained from eBiosciences (San Diego, CA, USA).

FACS analyses were performed on a Coulter EPICS XL (Beckman Coulter GMBH, Krefeld, Germany) or CyAn ADP Lx P8 (Beckman Coulter) flow cytometers. FACS data were analysed using FlowJo 8.8.3 software (Tree Star, inc. Ashland, USA).

Statistical analysis

Unpaired Students T test was used to calculate statistical significance.

6.4 Discussion

Advances in recent years have allowed researchers to investigate the hematopoietic system in even greater detail than before, and this was the origin for what we call the “full body stain” incorporating FACS stains of four different tissues. These stains are aimed at looking into the different stages of maturation of B-, T-, myeloid- and erythroid cells and were made available due to the published findings of groups that broke new ground in this field before we did so. This allows us to quickly assess if there is a hematopoietic disturbance in the mice investigated and to even possibly pin point where this alteration has its greatest effect.

In the *Sfrp1*^{-/-} mice we can here clearly show that mature hematopoietic cell populations of almost all categories, bar the T lymphocytes in the thymus, have been disturbed by the lack of Sfrp1.

The B cell compartment seems to be clearly affected by the loss of Sfrp1 with its more primitive cells showing a decrease and the more mature ones increased in proportion. There can be several explanations for this; one could be associated with the connection to estrogen, which speculates that estrogen can induce Sfrp1 production and this in turn leads to a reduction in lymphopoiesis. The study carried out by Yokota and co-workers used a fusion Sfrp1-Ig protein to study its effects on hematopoiesis, the results showed a reduction in B220+ cells in general and this can be clearly correlated to our own findings in which the B220 expressing cells are elevated in *Sfrp1*^{-/-} mice. However, this paper claims that the effect occurs in early lymphopoiesis which may very well be true but is not shown adequately in their experiments regarding the B cell lineage, which in the experiments involving Sfrp1 are only shown as B220 or Cd19 positive cells. But we here show is that in our

“whole body” stain the pro-B cells (B220+ Cd43+ IgM-) are in fact reduced in a *Sfrp1* deficient environment, in all tissues investigated, while in concurrence with the Yokota study the B220+, and also the more mature B cells (B220+/++ IgM+ Cd43-), are elevated. A depletion of earlier, more immature B cells and an increase in the more mature variety suggest that possibly the differentiation rate has been altered in some way and the fact that B220+ from *Sfrp1*^{-/-} cycle, according to BrdU measurements, more than their WT counterparts adds further strength to this argument (Renstrom et al., 2009). All of this could very well be related to estrogen but in our study we have not investigated this and studies looking at the levels of estrogen in *Sfrp1*^{-/-} mice could possibly shed some light on this matter, it would be interesting if the *Sfrp1* levels can affect estrogen levels or if it is always the other way around. Looking at the data at hand it would be tempting to suggest that in the *Sfrp1* Ig treated mice the B220+Cd43+IgM- population would be enlarged and that it would be here that *Sfrp1* inhibits B lymphopoiesis by blocking further differentiation. The proposed stabilization of β -catenin by *Sfrp1*, (Renstrom et al., 2009) and (Yokota et al., 2008), is most likely the reason for the alterations in the B cell and myeloid compartments seen in these *Sfrp1*^{-/-} mice, as mice with constitutionally active *Ctnnb1* display blocked hematopoietic differentiation pathways and these mice seem to exhibit the opposite in many ways (Kirstetter et al., 2006). But the mechanism which makes it so is not fully understood since previous papers, such as (Suzuki et al., 2004), argue that *Sfrp1* is a Wnt signalling antagonist and not an agonist as found here. One theory that could explain the stabilization of β -catenin by *Sfrp1* would be coupled to the findings of (Matsuyama et al., 2009), which states that *Sfrp1* interacts with *Wnt5a* a known canonical Wnt signalling inhibitor. But in order for this theory to make any sense one must also first consider the findings of (Uren et al., 2000) that suggest that *Sfrp1* can be both an agonist and an antagonist albeit at different concentrations. All these findings would fit together if it could be shown that *Sfrp1* has a higher affinity to bind to *Wnt5a* than to disturb Wnt signalling by binding to *Wnt3a* or similar canonical signalling agonists, then a lower concentration of *Sfrp1* could have a positive effect by reducing the effect of *Wnt5a* by binding to it. At the same time a higher concentration would turn the tables around with all available *Wnt5a* being bound up leaving the residual *Sfrp1* to act as an antagonist on the canonical Wnt pathway as is stated in many earlier publications. It could also partially explain the similarities with the *Wnt5a*^{-/-} mice and mice deficient in both *Sfrp1* and 2, assuming

that they are functionally redundant as stated by (Sato et al., 2006). This would have been a very interesting find but for which there was no time or funds for within the scope of this project.

Why T cells seemed unchanged in comparison to other cell populations could possibly be explained if *Sfrp1* is not expressed in the thymus, which is where the T cells mature in comparison to the other types of hematopoietic cells who predominantly develop in the bone marrow where *Sfrp1* is known to be expressed.

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Chapter 7: Efficient Hematopoietic Differentiation of Human Embryonic Stem Cells on Stromal Cells Derived from Hematopoietic Niches

Cell Stem Cell

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7.1 Summary

Hematopoietic stem cells derived from human embryonic stem cells (hESCs) could provide a therapeutic alternative to bone marrow transplants, but the efficiency of currently available derivation protocols is low. In this study, we investigated whether coculture with monolayers of cells derived from mouse AGM and fetal liver, or with stromal cell lines derived from these tissues, can enhance hESC hematopoietic differentiation. We found that under such conditions hESC-derived differentiating cells formed early hematopoietic progenitors, with a peak at day 18–21 of differentiation that corresponded to the highest CD34 expression. These hESC-derived hematopoietic cells were capable of primary and secondary hematopoietic engraftment into immunocompromised mice at substantially higher levels than described previously. Transcriptional and functional analysis identified *TGF- β 1* and *TGF- β 3* as positive enhancers of hESC hematopoietic differentiation that can further stimulate this process when added to the culture. Overall, our findings represent significant progress toward the goal of deriving functional hematopoietic stem cells from hESCs.

7.2 Introduction

Transplantation of hematopoietic stem cells (HSCs) in leukemia treatment requires high-quality sources of tissue-matched bone marrow, mobilized peripheral blood, or umbilical cord blood. Suitable bone marrow is often in short supply, and cord blood, although bankable, contains much lower numbers of HSCs, which makes it less suitable for adult transplantation. Directed differentiation of human embryonic stem cells (hESCs) toward HSCs offers a potentially attractive alternative to these conventional sources. The recent reports of somatic cell reprogramming by means of induced pluripotency makes this approach an even more exciting prospect for future cell-based therapies (Takahashi et al., 2007; Yu et al., 2007). Embryoid body (EB)-induced differentiation of hESCs can result in hematopoietic colony-forming cells (CFCs), and this can be further enhanced by BMP4 and a cocktail of cytokines, which is used for the expansion of bone marrow progenitor cells (Kennedy et al., 2007; Chadwick et al., 2003). Furthermore, it has been shown that hematopoiesis from hESC may arise from primitive endothelial-like cells that express *PECAM-1*, *FLK-1*

(*KDR*), and VE-Cadherin, suggesting the existence of an embryonic endothelium that possess hemangioblastic characteristics (Wang et al., 2004). Coculture of hESC with stromal cell lines such as S17 (Kaufman et al., 2001), OP9 (Vodyanik et al., 2005), and fetal liver-derived stromal cells (Qiu et al., 2005) can also result in spontaneous generation of hematopoietic cells; however, the overall extent of hematopoietic development is low (0.1%–2% of CD45+ during 8–20 days of differentiation). This is also reflected in vivo, where hESC-derived progenitors from coculture with S17 stromal cells engraft primary and secondary fetal sheep recipients with very low levels of chimerism (0.001%–0.05% of the bone marrow; Narayan et al. [2006]). The importance of embryonically derived HSCs has been thoroughly investigated in the murine embryo, showing that the first cells with long-term multilineage repopulation capacity that colonize the fetal liver and the bone marrow from day 10 of gestation arise in the aorta-gonad-mesonephros (AGM) region, which comprises the splanchnopleural mesoderm from the lower trunk and caudal region of the embryo (Medvinsky and Dzierzak, 1996; Cumanó et al., 2001). Culturing of the whole AGM region at an air/liquid interface results in a 15-fold increase in the number of HSCs, consistent with the hypothesis that this region produces factor(s) involved in the genesis, maintenance, and/or amplification of these cells (Medvinsky and Dzierzak, 1996). The work conducted in one of our groups indicates that coculture of murine ESC with primary AGM tissue in whole-organ culture and with a stromal cell line derived from that tissue results in enhanced hematopoietic differentiation (Krassowska et al., 2006). We show in this study that primary stromal cells derived from the AGM and fetal liver significantly enhance spontaneous hematopoietic differentiation in high-density hESC monolayer cell cultures. The hESC-derived hematopoietic cells were capable of primary and secondary hematopoietic engraftment into immunocompromised NOD/LtSz-Scid IL2Rg null recipients with up to 16% engraftment, which is significantly higher than described previously. Large-scale transcriptional analysis combined with signal blocking and over expression experiments led to identification of *TGF- β 1* and *TGF- β 3* as positive regulators of hESC differentiation in this system.

7.3 Results

7.3.1 Powerful Enhancement of Hematopoietic Differentiation of hESCs by Stromal Cells Derived from Hematopoietic Niches

One stromal cell line generated from the aorta/mesenchyme part of the AGM region of 10 dpc murine embryos (AM20.1B4), one from the urogenital ridge of the AGM region (UG26.1B6), and one from the fetal liver (EL08.1D2) of 11 dpc embryos were used in this study (Oostendorp et al., 2002a, 2002b, 2005a). We also investigated whether the primary stromal cells derived from the AGM (10.5 dpc) and fetal liver (12.5 dpc) region can enhance the hematopoietic differentiation of hESCs over a 25 day time period. For direct comparison, the experiments between stromal cell lines and primary cells were carried out in parallel and using the same early passage (35–50) hESCs. The stromal cells were mitotically inactivated and used in coculture experiments with hESCs over a 25 day differentiation time course monitored by flow cytometry and CFC assays. Pilot experiments with GFP-labelled hESCs cocultured with mitotically inactivated stromal cells and mitotically active stromal cells were carried out beforehand. This revealed no significant differences in the hematopoietic induction of hESC (data not shown); hence, mitotically inactivated stromal cells were used throughout this study. From day 12 to day 21, tight cobblestone patterns of cells appeared, and from day 18 onward differentiated cell types organized in “endothelial vessel” structures emerged (see Figures S1A and S1B, available online).

Differentiation of hESC was confirmed by the down regulation of two pluripotency markers, *OCT4* and *NANOG* (Figure S1C). CD34⁺ cells appeared at day 6 of culture and peaked on day 18 in all cases except when cocultured with the AM20.1B4 cell line (Figure 23A). The peak of CD34 expression corresponded to the maximum number of CFCs obtained (see below, Figure 24A). CD45⁺ cells appeared in small percentages in early stromal cell cocultures (from day 6, Figure 22B) and peaked by day 25; this occurs slightly later than in the cocultures with the primary stromal cell lines derived from the AGM region and fetal liver (Figure 23B). A small number of CD45⁺ cells were also identified in the control experiments in which hESCs differentiated in the absence of stromal feeder cells; however, this is most likely due to formation of a small number of EB structures resulting from the high culture density, especially at the later time points (our own observations). CD31⁺ cells first appeared on day 6 and peaked on day 12 in the stromal cell line cultures and on day

18 in the primary stromal cell cocultures (Figure 23C); however, the vast majority of them were CD34+CD31+ at these time points (Figures 23C and 23D). *KDR/VEGFR2* shows a biphasic expression pattern in the stromal cell line cultures (Figure 23E): an early expression peak between days 6 and 12 associated with CD34 expression and a later peak between days 18 and 21, not associated with CD34 expression (Figure 22F). This pattern is not observed in the primary stromal cell cocultures in which the peak of *KDR* expression at day 18 seems to correspond with that of CD34+KDR+ (Figures 23E and 23F). Using four-color flow cytometry analysis, we showed that 33.7% and 50.5% of CD34+KDR+ cells obtained from coculture of hESC with AGM or fetal liver primary cells, respectively (Figure S1D) were also CD31+VE-Cadherin+ consistent with the presence of hemangioblast-like cells suggested by Wang et al. (2004). A smaller percentage of such cells were also found in the cocultures with the stromal cell lines (Figure S1E). Hematopoietic CFC activity peaked at day 18 in all cases except coculture with the AM20.1B4 cell line concurrent with the peak of most hematopoietic markers described above (Figures 24A and 23A). A relatively low CFC activity was noticeable in the control group differentiated in the absence of any stromal cells by the end of differentiation process (days 21–25, Figure 24A), though this was significantly less than during coculture with the stromal cell lines and might be explained by the formation of EB-like structures with low hematopoietic activity within dense cocultures. As observed with murine ESC differentiation (Krassowska et al., 2006), coculture with stromal cells derived from primary AGM and FL regions resulted in a significant increase in hematopoietic activity when compared to a no-feeder control group. Representative colonies from the differentiation of hESCs showed typical morphology of various CFC subtypes, including macrophages, granulocytes, and erythrocytes as well as mixed colonies comprising different types of these cells (Figure 24B). Colony identity was confirmed by Wright-Giemsa staining of cytopsin preparations (Figure 24C). Analysis of CFCs from coculture with the stromal cell lines indicated a gradual decline in BFU-E and CFU-E from day 6 in all three coculture systems (hESCAM20.1B4, hESC-EL08.1D2, and hESC-UG26.1B6, Figure 24D). Cells within the CFU-G, CFU-M, and CFU-GM colonies were analyzed by flow cytometry and found to be CD45+ and coexpressing at least one of the following markers (CD64, CD33, and CD14), indicating early myeloid or granulocytic lineage commitment (Figures S2A–S2C). Erythroid cells were detected in mixed colonies by their hemoglobinization, glycophorin A expression, and the absence of

CD45 expression (Figure S2D). To prove these colonies were of human origin, DNA fingerprinting analysis was carried out on the hESCs and colonies recovered from methylcellulose assays (Figure S2E). This analysis, in addition to the complete absence of colonies arising from mitotically inactivated primary cells derived from the AGM region, fetal liver, or stromal cell lines, confirmed the human origin of hematopoietic colonies. Another observation was the presence of cells that lacked CD34 and CD45 expression but showed expression of some typical MSC markers during coculture with each of the three stromal cell lines as shown in Figure S3a-c.

7.3.2 Activation of Hematopoietic Gene Expression during hESC Coculture with AGM and Fetal Liver-Derived Stromal Cell Lines

To investigate in detail the hematopoietic differentiation of hESC on immortalized stromal cells, we carried out quantitative RT-PCR analysis for various genes that mark the development of mesoderm, and differentiation into hematopoietic precursors or more committed definitive hematopoietic cells. DNA sequences of oligonucleotides used in all PCR reactions were designed to amplify only the human ortholog. We found that expression of two mesodermal markers, *BRACHYURY* and *MIXL1*, was activated in the first 6 days of coculture, indicating formation of mesodermal cells preceding the emergence of early CD34+CD31+KDR+CD45_ precursors on day 12 (Figure 25A). Interestingly, the strongest activation of both genes occurred during coculture of hESC with the AM20.1B4 cell line, which also shows a later peak in CD34+ expression and CFC formation (Figures 24C and 25A). This suggests that AM20.1B4 is slightly more efficient in encouraging hESCs to form mesoderm and perhaps less-differentiated hematopoietic progenitor cells.

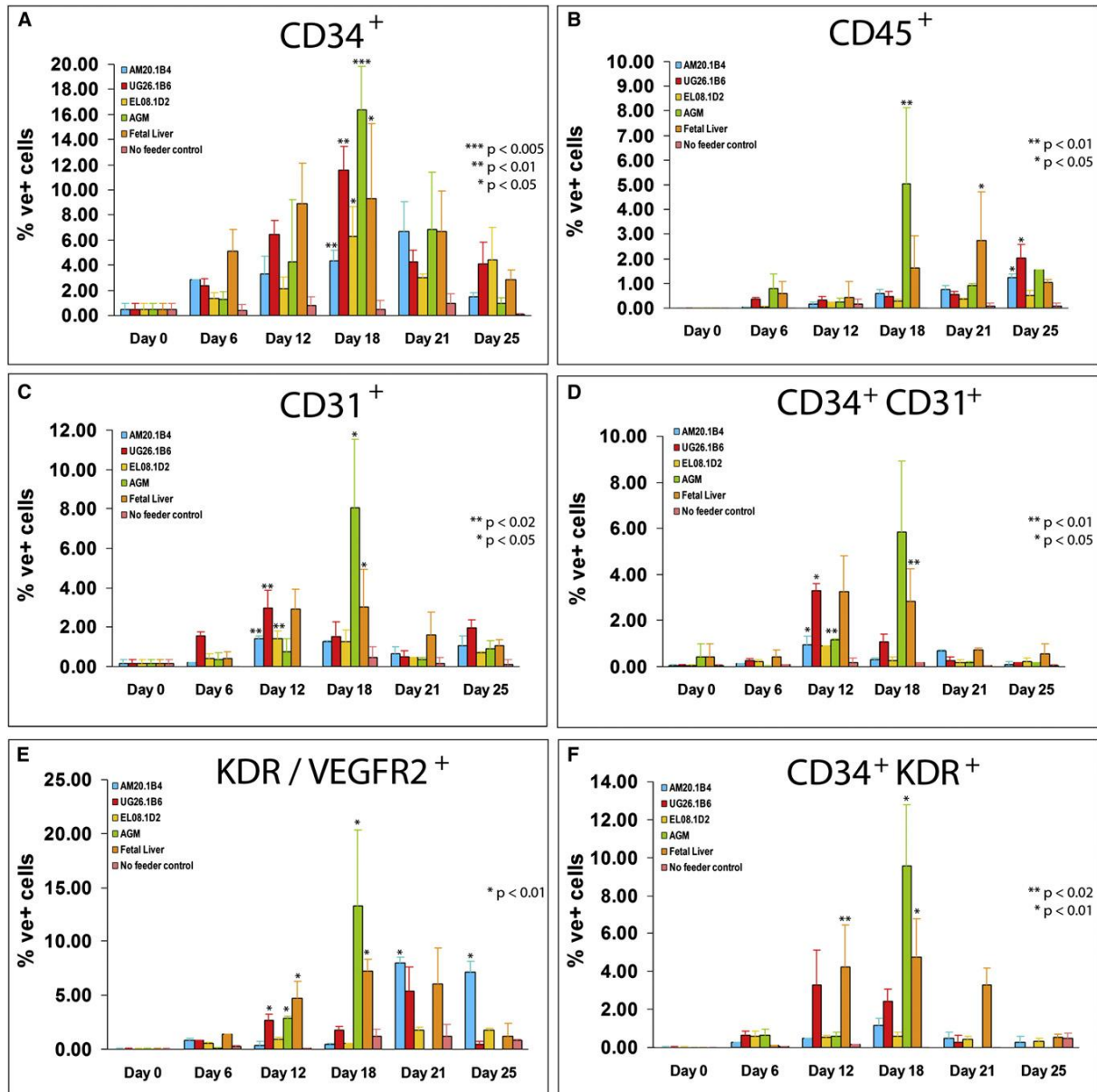


Figure 23. Kinetics of Hematopoietic Differentiation of hESCs Cocultured with Stromal Cells

(A–F) Kinetics of CD34⁺ (A), CD45⁺ (B), CD31⁺ (C), CD34⁺CD31⁺ (D), KDR⁺ (E), and CD34⁺KDR⁺ (F) during hESC differentiation, n = 3–6; error bars represent SD p values (for days on which peak expression was observed) for graphs shown in (A)–(F) and were calculated using arcsine transformation. All the antibodies used were tested to ensure specificity for the human antigens only.

This hypothesis is further supported by the observation that three important genes involved in early hematopoiesis in mice, *SCL* (Figure 25B) *GATA2*, and *LMO2* (data not shown), show the strongest upregulation during coculture with the AM20.1B4 cell line. Quantitative RT-PCR analysis for hemangioblast-related markers *KDR* and *CD31* (*PECAM1*) showed the strongest upregulation during coculture with the UG26.1B6 and EL08.1D2 cell lines at the early time points comprising days 6–18 (Figure 25B), which also correlates with the maximal CFC activity observed with these stromal cell lines (Figure 24A). Interestingly, *CDX4* activation was obvious on day 6 in the EL08.1D2 and UG26.1B6 cocultures followed by strong upregulation of *HOXB4* from day 12 onward (Figure 25B), suggesting the onset of definitive hematopoiesis in these two cocultures. Additionally, the expression of *RUNX1/AML* and *PU.1* that is important for definitive hematopoietic cell differentiation was best up regulated in the UG26.1B6 coculture on day 18, which correlates with peak CFC activity and CD34 expression in these cocultures (Figure 25B). Erythroid-like cells derived from hESCs coexpress high levels of embryonic and fetal globins but little or no adult globins (Chang et al., 2006). This necessitates the investigation of different globin expression in erythroid cells obtained after CFC assays. The specificity of each primer pair was investigated by

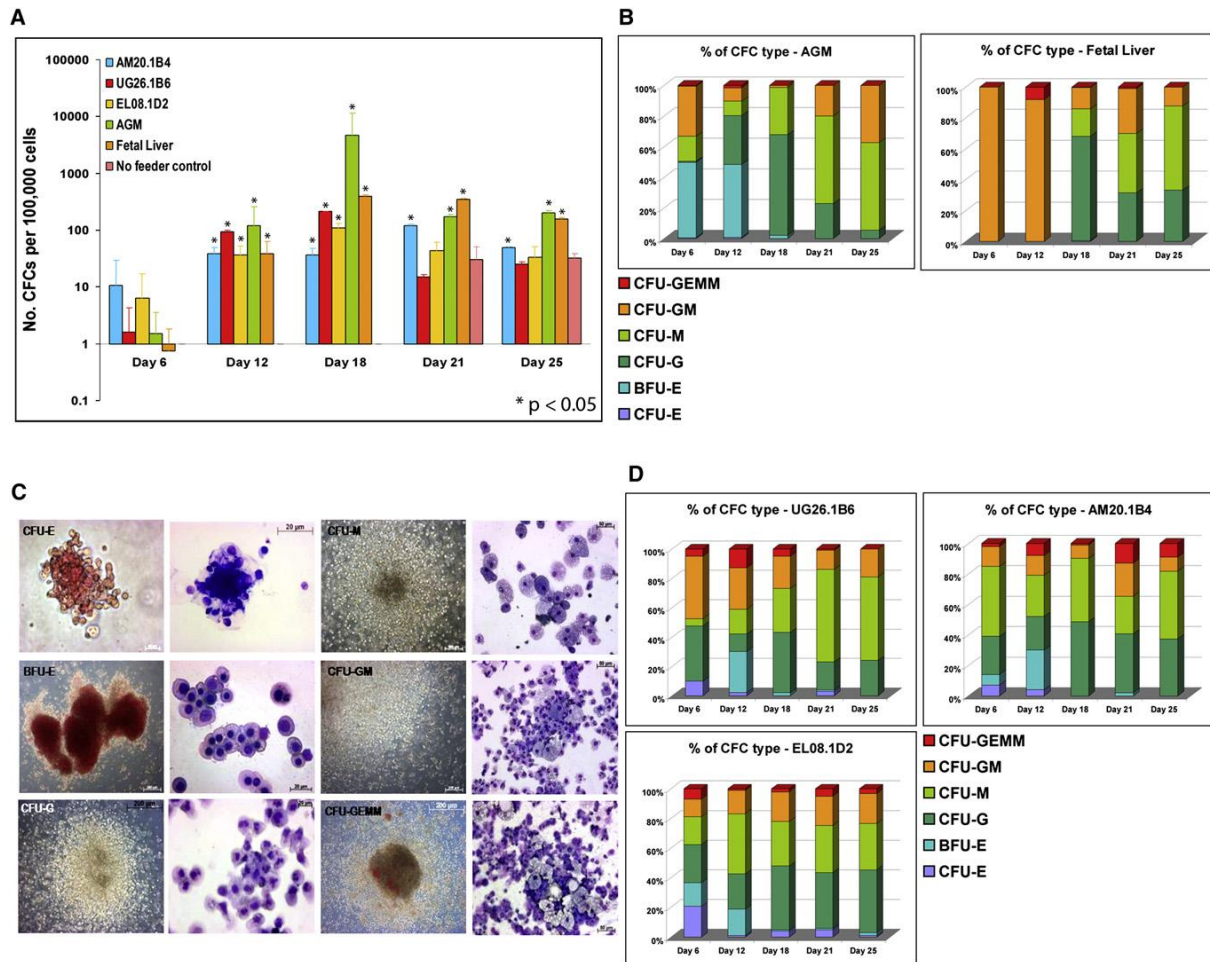


Figure 24. Hematopoietic Progenitor Capacity of hESCs Differentiating on Stromal Cells

(A) CFC activity of hESC differentiating on stromal cells as compared to no-feeder control (n = 4–8), error bars represent SD. Significance between each coculture and no-feeder control was calculated using Student’s t test. (B) Distribution of hematopoietic colony subtypes obtained under differentiation of hESC on primary stromal cells derived from the AGM region and the fetal liver (n = 4–8). (C) Wright-Giemsa stains of cytopsin preparations of erythroid colonies (CFU-E and BFU-E), granulocytic (CFU-G), macrophage (CFU-M), and mixed colonies (CFU-GM and CFU-GEMM). (D) Distribution of hematopoietic colony subtypes obtained under differentiation of hESC on stromal cell lines.

using human bone marrow and cDNA from an early human embryo as positive controls; therefore, the lack of any PCR product in the cells obtained from CFU-E/BFU-E would indicate lack in expression rather than failure in the PCR procedure. It is well known that coordinated switching of the α -like and β -like genes results in the expression of embryonic Hb Gower-1 ($\alpha 2\beta 2$), fetal Hb-F ($\alpha 2\gamma 2$), and adult Hb-A ($\alpha 2\beta 2$). Additionally, there are other structurally defined hemoglobins such as Gower-2 ($\alpha 2\beta 2$), Portland-1 ($\alpha 2\gamma 2$), and Portland-2 ($\alpha 2\beta 2$), which are expressed at low levels in primitive and definitive erythroid cells primarily during fetal and embryonic

development. Expression analysis of these possible globin combinations in CFU-E colonies indicated that erythroid progenitors generated in the first 12 days of differentiation are likely to express Gower-1, fetal Hb-F, and Portland-1 (Figure 25C); however, from day 12 onward, only the Gower-2 form can be produced. The same analysis on CFU-Es taken from different days of differentiation showed a reduction in the expression of $\alpha 2$ and $\beta 2$ chains and therefore little expression of the embryonic Gower-1, Portland-1, and Hb-F, which suggests that Gower-2 was the predominant combination in the first 18 days, while fetal Hb-F and Gower-2 were coexpressed from day 18 onward (Figure 25C).

7.3.3 The Hematopoietic Induction Effect Is Not Entirely Retained in the Conditioned Media

To understand the nature of the induction events, we separated the two cell types using transwell inserts. Flow cytometry analysis showed large reductions in hematoendothelial cell surface marker expression (data not shown). Interestingly, the total number of CFCs was significantly reduced when transwell inserts were used in combination with UG26.1B6 and EL08.1D2 cell lines (Figure 25A), due to the complete absence of CFU-E and BFU-E and significant reduction in other CFC types in both cell lines. In contrast, the conditioned medium from the AM20.1B4 cell line resulted in the maintenance of CFC activity with the majority of colonies being of the CFU-M type (Figure 25A). A significant reduction in hematopoietic cell surface marker expression and lack of CFC activity was also noted when transwell inserts were used during the coculture of hESC with primary stromal cells derived from 10.5 dpc AGM and 12.5 dpc fetal liver region, with CFCs from these experiments giving rise only to very few CFU-M on day 12, resulting in 98% loss of hematopoietic CFC activity (data not shown).

7.3.4 The Hematopoietic Induction Effect Is Not Entirely Retained in the Extracellular Matrix of the Stromal Cell Lines

To investigate whether the extracellular matrix (ECM) extracted from the stromal cell lines retains their hematopoietic inducing activities, we isolated the pericellular matrix from the cells using deoxycholate and cultured hESC on this matrix for 18 days in the presence of FCS-containing media (ECM+FCS medium, Figures 26B–26D). In all

three cases, CFC capability declined; however, there were large variances between the cell lines with AM20.1B4 retaining 30% of the CFC activity, while EL08.1D2 and UG26.1B6 retained only 2% and 7%, respectively (Figures 26B–26D). When the FCS-containing medium was conditioned by the cells and combined with ECM, hematopoietic activity was lost for UG26.1B6 and EL08.1D2 (Figures 26C and 26D). In the case of AM20.1B4, we noticed more terminal differentiation (only CFU-M was observed instead of CFU-G, M, and CFUGM) for FCS-conditioned medium combined with the ECM of this cell line, which again correlates with the transwell data showing that CFU-M was the predominant colony type formed (Figure 26B). To better define the culture conditions for hematopoietic differentiation of hESCs, we cultured these cells on ECM derived from each of the cell lines in serum-free medium (stem line, Sigma) supplemented with three human cytokines (SCF, TPO, FLT-3L) or five human cytokines (BMP4, SCF, FLT-3L, TPO, and VEGF-A165) as described by Tian et al. (2004). Addition of the three cytokines to the serum-free medium in combination with the ECM derived from the stromal cell lines resulted in complete abrogation of the hematopoietic activity in the AM20.1B4 cell line and retention of only 36% and 6.4% of this activity in the EL08.1D2 and UG26.1B6 cell lines, respectively (Figures 26C and 26D). Supplementation of the serum-free media with five cytokines and in combination with ECM abolished hematopoietic CFC activity of all three stromal cell lines (Figures 26B–26D), corroborating previous data by Tian et al. (2004). Similar experiments carried out with ECM extracted from primary AGM or FL stromal cells showed a total loss of hematopoietic CFC activity, indicating that direct cell-to-cell contact was crucial for ESC differentiation on primary stromal cells.

7.3.5 Investigation of Molecular Pathways Involved in the Hematopoietic Inductive Effect

To better define the molecular mechanisms modulating the hematopoietic differentiation of hESC cocultured with stromal cells, potential hematopoietic signalling pathways were modulated. Gene expression analyses from macroarray (Oostendorp et al., 2005b) and microarray data (Figure S4) of the three murine stromal cell lines used in this study as well as a number of documented hematopoietic regulators (*VEGFR2*, *SDF1*, *IL6*, *M-CSF*, *TPO*, and *GM-CSF*) were taken together to produce a list of candidates, which might mediate hematopoietic induction. Cocultures with UG26.1B6 were used for these experiments, since these

produced the highest number of CD45+ cells when compared to the other two stromal cell lines (Figure 23B). Chemical or antibody inhibitors for these factors were added to the differentiation media for 18 days at optimal inhibitory concentrations (Table S1). Since the expression of CD45 closely correlated to the CFC activity, flow cytometry analysis for this marker was used to identify possible effects on the hematopoietic differentiation of hESCs. Several of the identified candidates including members of the FGF family (*FGF 7* and *FGF 9*), members of the IGF family (*IGF2*), and IGF binding proteins (*IGFBP3* and *IGFBP 4*), cytokines (*IL6*, *IL3*, *IL1*, *MCSF*, *GM-CSF*, *TNF α*), chemokines (*CCL2*, *CCL5*, *SDF1*), and in particular candidates linked to the TGF- β superfamily of signalling factors (*TGF- β 1–3*, Activin, *BMP4*, and *BMP2*) modulate the induction of CD45+ cells from hESC. Both stimulating and inhibitory activities were found with the TGF- β family members. Inhibition of the Activin receptor 1b (*ALK4*), TGF- β receptor 1 (*ALK5*), and Nodal receptor 1 (*ALK7*) by ALK 4-5-7 resulted in the most significant changes in cell morphology (data not shown) and decrease of hematopoietic marker expression during differentiation, whereas inhibition of *BMP4* alone, *BMP4* and *BMP2* together (via Gremlin), Activin receptor type 1b (*ALK4*), and TGF- β 2 via binding of TGF- β soluble receptor III (betaglycan) resulted in a significant increase in CD45 expression (Table S1), suggesting that these later factors were unlikely to account for the significant decrease in hematopoietic commitment observed upon application of ALK 4-5-7.

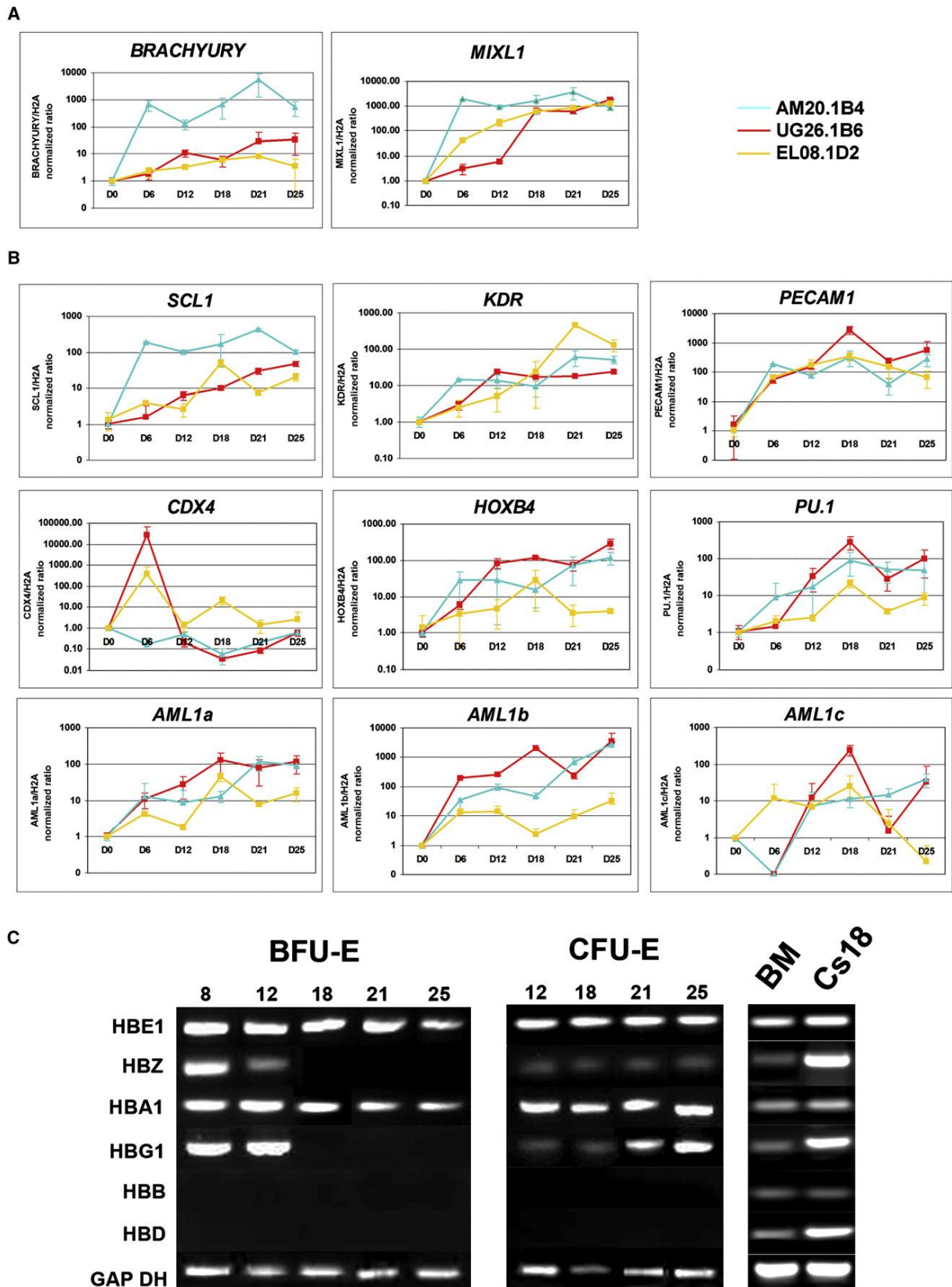


Figure 25. Expression of Mesodermal, Hematopoietic, Endothelial, and Homing Markers during hESC Differentiation (A and B) Quantitative RT-PCR analysis for the expression of mesodermal markers (A) and hematopoietic and endothelial markers (B). (C) RT-PCR analysis for the expression of embryonic, fetal, and adult globins in cells taken from BFU-E and CFU-E colonies obtained after

differentiation of hESC on EL08.1D2 stromal cells (similar results were obtained with UG26.1B6 and AM20.1B4 stromal cell lines). In all cases, oligonucleotides were designed with the aim of distinguishing the human from the mouse gene, and control PCRs on murine stromal cell lines were carried out to ensure amplification of human ortholog only. Human bone marrow (BM) and human embryo (Carnegie stage 18, Cs18) cDNA were used as positive controls. The data are presented as average \pm SD, $n = 3$.

HBE1 denotes the β chain, HBZ denotes the γ chain, HBA1 denotes the α chain, HBG1 denotes the δ chain, HBB denotes the β chain, and HBD denotes the δ chain.

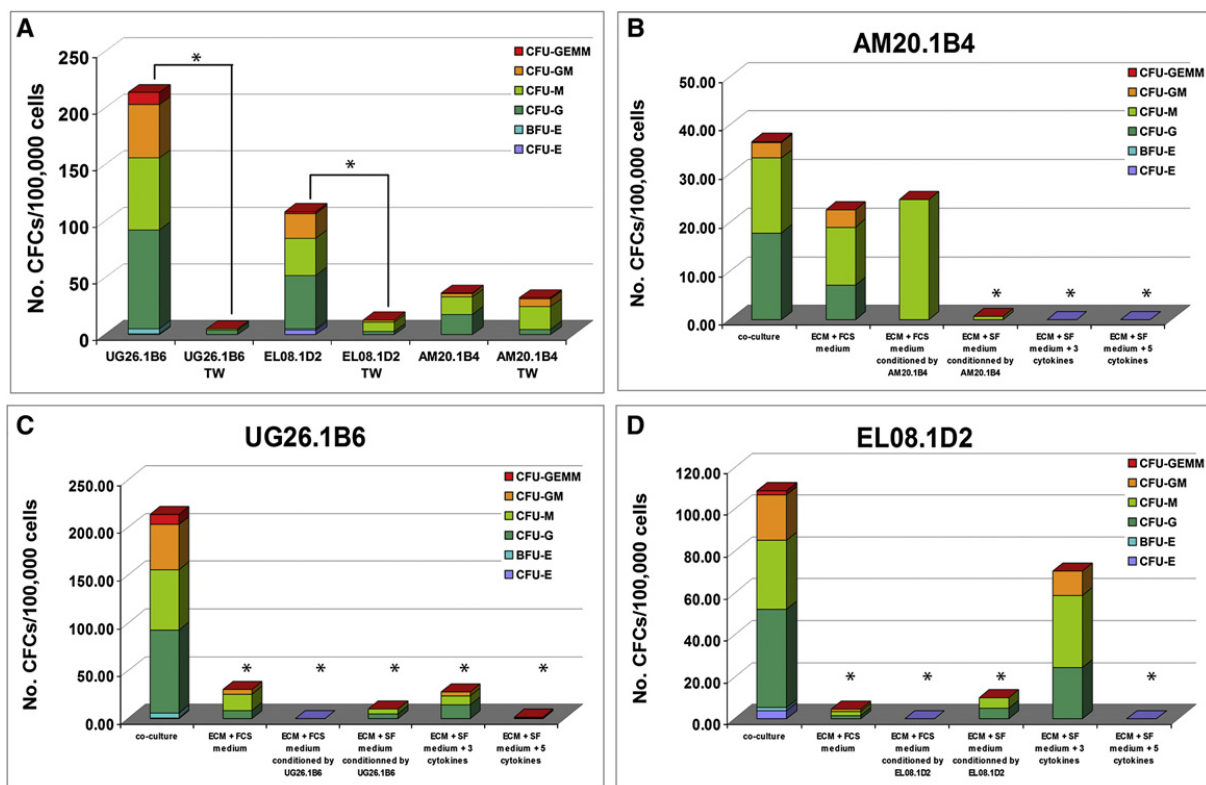


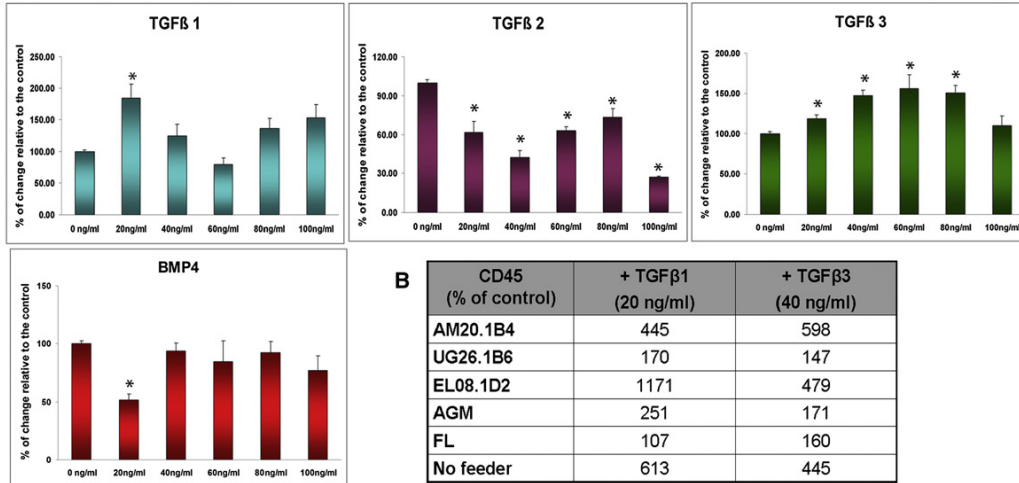
Figure 26. Hematopoietic Activity Is Not Entirely Retained in the Conditioned Media or the ECM of the Stromal Cell Lines

(A) Hematopoietic CFC activity in the presence and absence of transwell insets at day 18 of hESC differentiation on stromal cell lines. Significance between normal coculture and transwell inset culture was assessed by Student's t test, $p < 0.05$. (B–D) Hematopoietic CFC activity of hESC after 18 days of differentiation on ECM isolated from the AM20.1B4 stromal cell line (B), UG26.1B6 (C), and EL08.1D2 (D) and in the presence of serum-free medium and cocktails of three (SCF, TPO, and FLT-3L) or five (BMP4, SCF, VEGF-A165, FLT-3L, and TPO) cytokines.

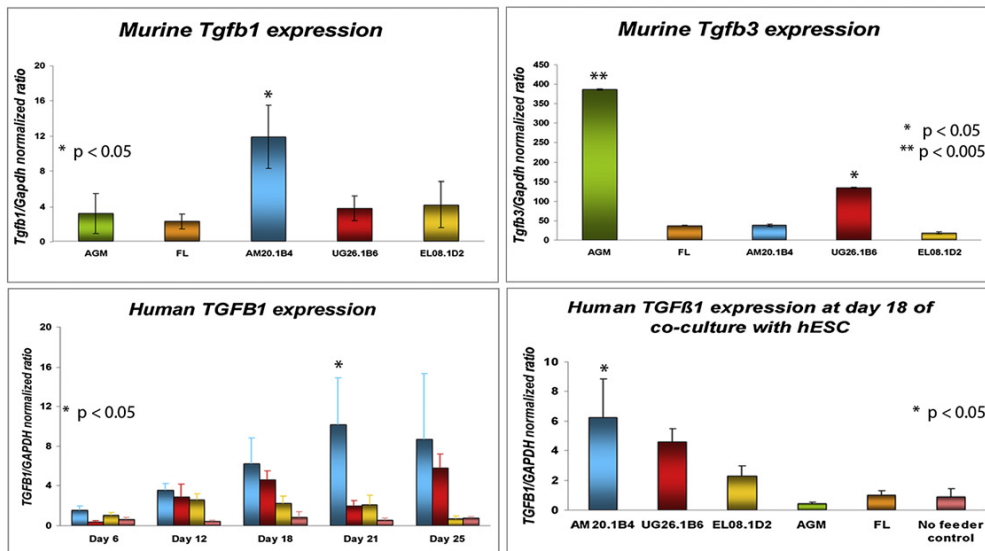
Significance between normal cocultures and new methods used to induce hESC differentiation was calculated by using Student's t test

Instead, inhibition of TGF- β 1 and TGF- β 3 alone resulted in a significant decrease in CD45 expression (Table S1). To investigate the role of some of the TGF- β family members in more detail, we added to our coculture system human soluble TGF- β 1, -2, and -3, BMP4 (Figure 27A), and Activin A (data not shown) in a dose-dependent manner. This showed that both TGF- β 1 and TGF- β 3 enhance hematopoietic differentiation of hESC cocultured with the UG26.1B6 cell line most effectively at different concentrations (TGF- β 1, 20 ng/ml; TGF- β 3, 20–80 ng/ml; Figure 27A). In contrast, TGF- β 2 (20–100 ng/ml), BMP4 (20 ng/ml; Figure 5A), and Activin A (1–30 ng/ml; data not shown) reduced CD45 expression, pointing to a negative role for these factors in hematopoietic commitment of hESCs in this coculture system when added for the whole length of differentiation period (Figure 27A). Addition of soluble human TGF- β 1 or TGF- β 3 to differentiating hESCs cocultured in the presence or absence of the other stromal cell lines or primary cells indicated a significant increase in hematopoietic commitment of hESCs (Figure 27B), suggesting that addition of these two growth factors is beneficial to their differentiation. Expression analysis of a number of TGF and BMP family members, receptors, and modulators (Figure S4) indicated that *Tgfb1* is expressed at higher levels in the AM20.1B4 compared to UG26.1B6 and EL08.1D2. Quantitative RT-PCR analysis confirmed that *Tgfb1* was most highly expressed in AM20.1B4 compared to the other stromal cell lines and primary cells derived from the AGM and FL region (Figure 27C). In contrast, highest *Tgfb3* expression was seen in primary cells from the AGM region and the UG26.1B6 cell line (Figure 27C). A similar analysis for human *TGF β 1* and *TGF β 3* expression indicated a gradual increase in their expression during the differentiation time course (Figures 27C and 27D).

A



C



D

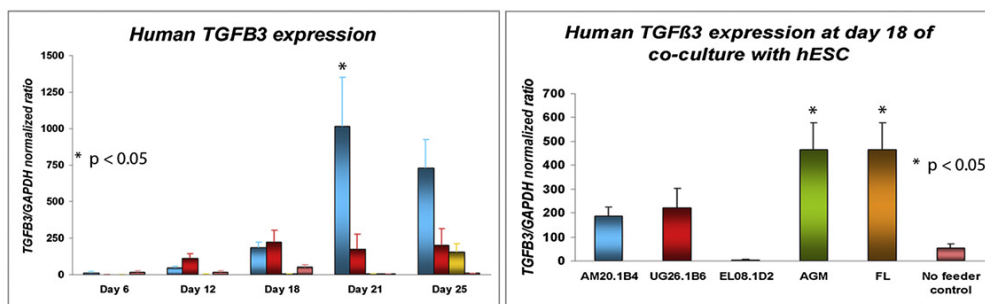


Figure 27. Modulation of Hematopoietic Signalling during hESC Differentiation on Stromal Cell Lines

(A) The effects of TGF- β 1, TGF- β 2, TGF- β 3, and BMP4 on hematopoietic differentiation of hESCs cocultured with the UG26.1B6 stromal cell line measured by changes in CD45 expression. The data are presented as average \pm SD, $n = 3$. Significance between each sample and the control (0 ng/ml) was calculated by using Student's t test. (B) The effects of TGF- β 1 and TGF- β 3 on hematopoietic differentiation of hESCs cocultured in the presence or absence of stromal cell lines and primary cells derived from the AGM and FL region measured by changes in CD45 expression. The data are presented as the average of three independent experiments (C and D) Quantitative RT-PCR for the expression of murine *Tgfb1* and *Tgfb3* in murine stromal cells and human *TGF β 1* and *TGF β 3* during

hESC differentiation in the presence of stromal cells. The data are presented as average \pm SD, n = 3. P values were calculated by using arcsine transformation. Oligonucleotides used in the RT-PCR reaction were checked to ensure amplification of species-specific *Tgfb1* and *Tgfb3* only

Similar investigations carried out with hESCs cocultured with primary stromal cells derived from the AGM and FL region indicated a steady increase in the expression of human *TGFb3* and *TGFb1* during differentiation, similar to stromal cell lines (data not shown). Very low expression of these genes was observed in differentiating hESC in the absence of stromal cells (Figures 27C and 27D), suggesting that stromal cells per se might be important for induction of endogenous *TGFb1* and *TGFb3* expression during hESC differentiation. Highest *TGFb1* and *TGFb3* expression was observed in differentiating hESCs that were cocultured with the AM20.1B4 cell line and primary stromal cells obtained from the AGM/FL region, respectively (see Figures 27C and 27D for one example on day 18 of coculture with hESC). This correlates well with the highest expression of murine *Tgfb1* and *Tgfb3* in the AM20.1B4 and AGM-derived stromal cells, respectively, suggesting that feeder-produced *Tgfb1* and *Tgfb3* might influence the production of endogenous human *TGFb1* and *TGFb3* during hESC differentiation, although this needs to be investigated further.

7.3.6 Detection of hESC-Derived Hematopoietic Progenitors in the Hematopoietic System of NOD/LtSz-Scid IL2Rg null Recipients

Approximately 5.3×10^5 cells derived from coculture of hESC with the three stromal cell lines and primary stromal cells generated from the AGM and FL region were injected intrafemorally into NOD/LtSz-Scid IL2Rg null recipients 24 hr after total-body irradiation. As an additional control, hESC differentiated in the absence of any stromal cells were transplanted into this murine model. Our results show the presence of hESC-derived MSCs (Figure S3) that might have a beneficial role on the engraftment of hESC-derived HSCs. For this reason, we transplanted the whole cell mixture at 12 (stromal cell line cocultures) and 18 days (primary cell line cocultures) of differentiation shown to correspond to peak hematopoietic progenitor activity by coexpression of CD34 and CD31 (Figure 23D). Eight to 12 weeks after transplantation, the mice were sacrificed, and their peripheral blood and bone marrow from the injected and uninjected femur were analyzed by flow cytometry for the presence of human CD45+ cells. Vibrant violet dye was used to distinguish cells from

debris while human peripheral blood, mouse peripheral blood, and human peripheral blood spiked with a small amount of mouse peripheral blood were used as controls to set up flow cytometry gates for human and mouse CD45+ cells, respectively (Figure 28A). All surviving mice demonstrated human reconstitution in peripheral blood and injected and uninjected femurs (Figure 28B). Of interest is the observation that hESC-derived cells from coculture with the AM20.1B4, UG26.1B6 primary cells from the AGM and FL region had better survival efficiency (Figure 28B), since more mice engrafted with these cells (6/9, 6/9, 6/6, and 7/7, respectively) compared to mice injected with hESC differentiated in the presence of EL08.1D2 cell line (only 2 out of 12 mice engrafted and survived). In addition, there was a higher percentage of human CD45+ cells in the peripheral blood of mice engrafted with hESC differentiated in the presence of AM20.1B4 when compared to the UG26.1B6 cell line ($p = 0.0129$) or primary cells from the AGM ($p = 0.0238$) or fetal liver region ($p = 0.0012$). The mice engrafted with hESC differentiated in the presence of AM20.1B4 cell line also showed a significantly higher percentage of CD45+ cells in the left femur ($p = 0.035$), right femur ($p = 0.051$), and peripheral blood ($p = 0.0022$) when compared to mice engrafted with hESCs differentiated in the absence of feeder cells (Figures 28B and 28C). Significant differences in engraftment of human CD45+ cells in the right and left femurs were also observed when mice transplanted with hESC differentiated in absence of any feeder layers were compared to hESC cocultured with cells derived from primary AGM and fetal liver region ($p < 0.005$ in all cases), thus highlighting the role of these feeder cells in enhancing the in vivo engraftment of hESC-derived hematopoietic cells. In all cases, there were no statistically significant differences in engraftment efficiencies between the left and the right femur (Figure 28B). The flow cytometry results were confirmed by PCR of genomic DNA obtained from the femurs, spleen, and peripheral blood of injected mice using human chromosome 17-specific satellite sequences (Figure 28B). Interestingly, there were no significant differences between the mean engraftment efficiency of human CD45+ in the mice transplanted with hESC differentiated in the presence of primary AGM-derived cells compared to the mice transplanted with hESC that were cocultured with UG26.1B6 or AM20.1B4 in the right and left femur (Figures 28B and 28C). In contrast, there was a significantly higher percentage of CD45+ cells in the left and right femur of mice engrafted with hESCs differentiated in the presence of fetal liver cells compared to those cocultured with EL08.1D2 cell line ($p = 0.0061$). The

difference between these two experimental observations can relate to the very low engraftment efficiency achieved by differentiating hESC cocultured with EL08.1D2, which makes the difference between the primary stromal cells and the cell line much more significant. The human CD45+ cells from uninjected femurs and peripheral blood were sorted using FACS Aria (BD Biosciences) and analyzed for expression of myeloid and lymphoid markers (Figure 28D). The coexpression of human CD13 and CD33 indicated the presence of hESC-derived myeloid cells. CD2 and CD19 are also present on these cells, suggestive of hESC-derived T and B cells in the primary mouse recipients. It is known that CD2 is expressed in up to one third of circulating monocytes and myeloid dendritic cells in addition to being expressed in T cells, while CD19 can also be expressed in monoblasts and early myeloid progenitor cells; thus, single expression of these markers, although suggestive, cannot be taken as definite proof of lymphoid engraftment in this animal model.

Secondary transplants were carried out by taking $2,3 \times 10^6$ cells from the bone marrow of primary recipients (engrafted with hESC differentiated in the presence of UG26.1B6 cell line) and injecting them into the femurs of secondary recipients (Figure 28E). The flow cytometry analysis and PCR analysis confirmed the presence of hESC-derived human cells in all surviving secondary recipients analyzed, thereby confirming the presence of hESC derived HSCs in our cocultures.

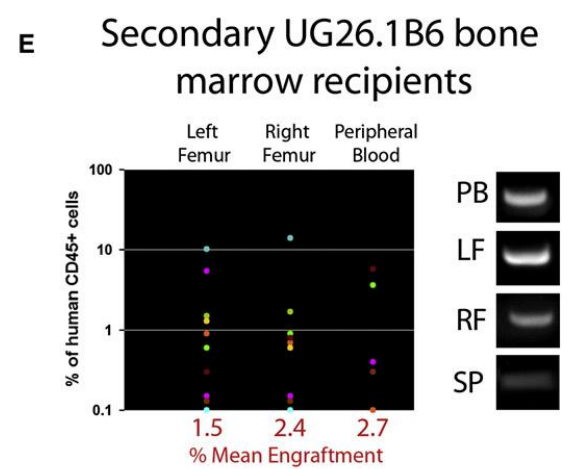
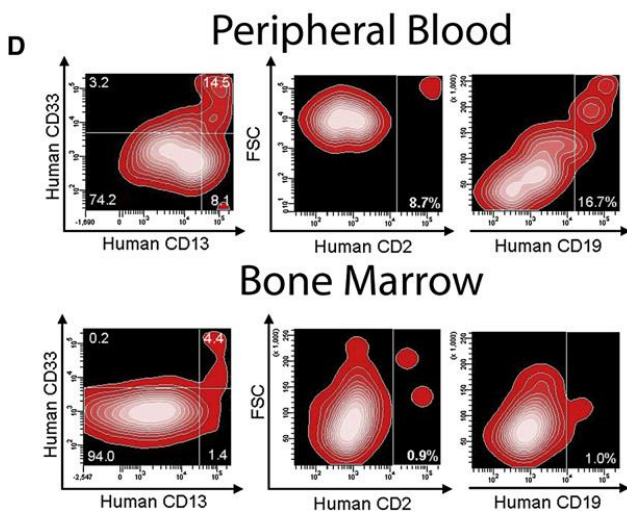
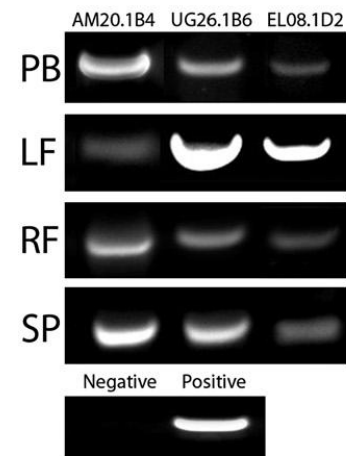
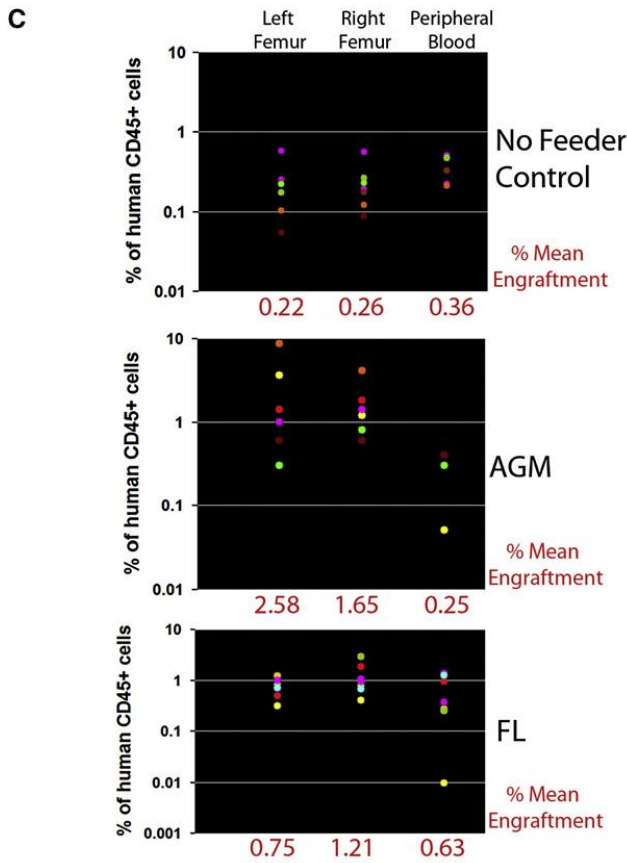
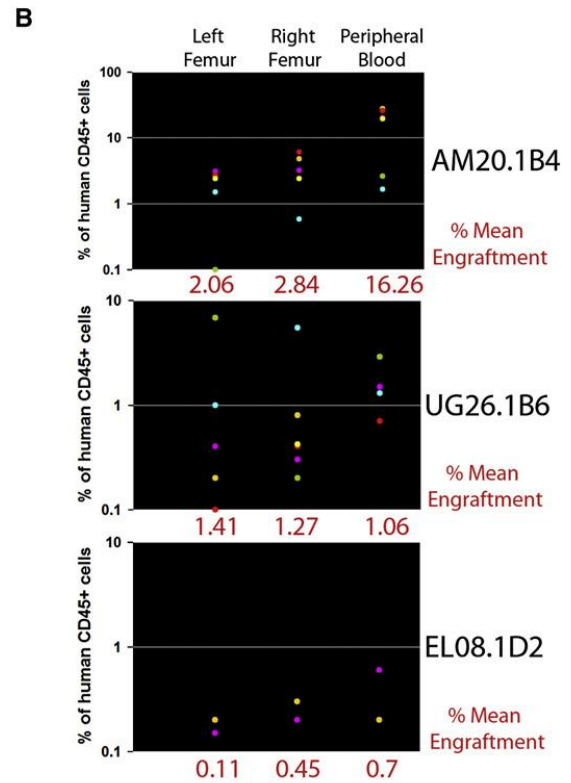
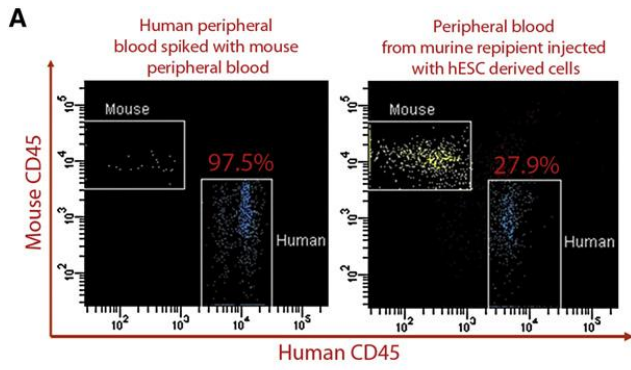


Figure 28. Detection of hESC-Derived Cells in the Hematopoietic System of Sublethally Irradiated NOD/LtSz-Scid IL2Rgnull Recipients

(A) Flow cytometry analysis for detection of human and mouse CD45+ cells in human peripheral blood spiked with mouse peripheral blood (left panel) and the peripheral blood of NOD/LtSz-Scid IL2Rgnull recipients that have been transplanted with hESC differentiated cells (right panel). (B) Detection of hESC-derived cells in the left femur, right femur, peripheral blood, and/or spleen of primary mouse recipients that were injected with hESC differentiated in the presence of UG26.1B6, AM20.1B4, and EL08.1D2 by PCR (top panel) and flow cytometry (bottom panel). Six out of nine mice showed hESC-derived cells from coculture with AM20.1B4, and 6 out of 9 mice showed hESC-derived cells from coculture with UG26.1B6, while only 2 out of 12 mice showed hESC-derived cells from coculture with EL08.1D2. PB, peripheral blood; LF, left femur; RF, right femur; and SP, spleen. Mann-Whitney U test was used to evaluate the differences in the engraftment efficiencies between mice engrafted with hESC differentiated in the presence of three stromal cell lines. (C) Detection of hESC-derived cells in the left femur, right femur, and peripheral blood of primary mouse recipients that were injected with hESC differentiated in the presence of primary stromal cells derived from the AGM and fetal liver region as well as absence of any feeder layers. PB, peripheral blood; LF, left femur; RF, right femur; and SP, spleen. Mann-Whitney U test was used to evaluate the differences in the engraftment efficiencies between mice engrafted with hESC differentiated in the presence or absence of various stromal cell lines. (D) Flow cytometry analysis of peripheral blood and bone marrow from primary recipient that had received hESC cells differentiated in the presence of AM20.1B4 for presence of human-derived myeloid and lymphoid-like cells. (E) Detection of hESC-derived cells into secondary recipients that received bone marrow transplants from primary mice injected with hESC-derived cells differentiated in the presence of UG26.1B6 cell line by flow cytometry (right panel) or PCR (left panel). Ten out of twenty mice showed engraftment with hESC-derived cells. In all cases, each individual mouse is indicated by a colored circle.

7.4 Discussion

Our previous work has shown that coculture of murine ESC with intact primary AGM region resulted in a significant increase in hematopoietic colony formation (Krassowska et al., 2006). For the first time, we show that the coculture of hESC with primary stromal cells derived from the AGM region enhances their hematopoietic differentiation. Of particular significance has been the much higher efficiency of hematopoietic activity generated after the exposure of hESC to the primary cell lines derived from the AGM region (at least 31-fold) compared to hematopoietic activity induced by previously published methods, including the EB differentiation method, and by coculture with OP9 stromal cells. Various coculture methods used in this study have allowed us to enhance the in vitro differentiation of hESC and make several observations as outlined below. First, exposure of hESC to primary stromal

cells derived from the AGM and fetal liver results in at least 22- and 12-fold more CFCs, respectively, compared to coculture with stromal cell lines derived from these regions. This suggests that the coculture with the primary stromal cells is more conducive to proliferation or induction of the type of hematopoietic progenitor cells detectable by the in vitro CFC assay. However, this might not necessarily be the most primitive HSC that can be detected by in vivo assays, since this cell type is normally quiescent under steady-state hematopoiesis. Second, the UG26.1B6 cell line was the most efficient at enhancing hematopoietic activity in vitro compared to the other cell lines, AM20.1B4 and EL08.1D2. This suggests that the UG26.1B6 cell line has the capacity to maintain hematopoietic progenitors and perhaps enhance their proliferation rather than induce hematopoietic differentiation per se. Third, transplantation into immunocompromised recipients shows that AM20.1B4 is the best cell line for generating a significant number of circulating hESC derived hematopoietic progenitors compared to the UG26.2B6, EL08.1D2, or primary stromal cells derived from the AGM and fetal liver region, thus highlighting differences in results obtained from in vitro and in vivo work. This could be explained by subtle differences between stromal cell lines such as the ability to induce differentiation and generate more primitive precursor cells (perhaps applicable to the AM20.1B4 cell line) versus the ability to induce the proliferation of existing progenitors (as in the case of UG26.2B6 cell line and primary stromal cells). The second influencing factor could be the difference in the progenitor cell type(s) being detected by in vitro assays versus in vivo work. This hypothesis is supported by our previous work in which HPP-CFC and CFU-A assays (suggested to identify more primitive progenitors than CFU-GEMM) identified AM20.1B4 as being most efficient in inducing the differentiation of most primitive hematopoietic progenitors from murine ESC (Krassowska et al., 2006). Differentiation by coculture with the different stromal cell lines might also have a significant effect on the in vivo survival, homing, and differentiation ability of hematopoietic progenitor cells. Although the primary stromal cells might generate highly prolific hematopoietic progenitor cells, their homing to the recipient's bone marrow and subsequent differentiation might not be as efficient as more primitive progenitor cells that may be produced by AM20.1B4 cocultures. Our functional screen for potential hematopoietic modifiers identified a positive role for *TGF- β 1* and *TGF- β 3* in enhancing hESC differentiation to hematopoietic lineages and implied a negative role for *BMP4*, *TGF- β 2*, and *Activin A*. It is well established that *BMP4* plays

a critical role in the specification of hematopoietic cells from the mesoderm in vivo (Bhatia et al., 1999), can modulate the proliferation and lineage potential of AGM derived CD34+ cells (Marshall et al., 2007), and is important for enhanced hematopoietic differentiation of hESCs in vitro (Wang et al., 2004; Tian et al., 2004). The role of *BMP4* has been investigated in murine ESC showing that *BMP4* is required for fate specification (posterior) within the primitive streak and induction of Flk1+ mesoderm; however, it is not required for primitive streak formation and specification of Flk1+ mesoderm to the hematopoietic lineages (Nostro et al., 2008). Recent data have suggested that only short-term *BMP4* treatment is needed to initiate mesoderm induction in hESCs, and this capacity is dependent on the presence of endogenous FGF and the TGF- β /Nodal/Activin signalling pathway (Zhang et al., 2007). Longer *BMP4* treatment can result in trophoblast and extraembryonic endoderm differentiation of hESCs. Our results corroborate published findings and show that long treatments with *BMP4* do indeed suppress hESC differentiation via the stromal coculture method. *TGF- β 1* is also known to regulate HSC proliferation and pool size in the adult blood system (Bhatia et al., 1999), has been shown to regulate G1/S cell-cycle progression and expression of c-Kit to maintain a primitive and undifferentiated population of HSC progenitors (Bhatia et al., 1999), and can activate *Smad 5* (which is also regulated by *BMP4*; Ruscetti et al. (2005)). It is known that TGF- β isoforms perform distinct functions in the adult and throughout embryogenesis, although data elucidating these functions are still emerging and there are few data relating to the function of TGF- β isoforms in ESC differentiation. Studies in murine ESC have shown that endoglin, an ancillary reporter for the TGF- β family, is required for hemangioblast and early hematopoietic development (Perlingeiro, 2007) and that a close interplay between *VEGF*, *TGF- β 1*, and Activin A signalling is required for the generation of hematopoietic and endothelial cells from murine ESC (Park et al., 2004). Recent data suggest that TGF- β signalling in HSCs is modulated at multiple levels by *HOXB4*, which has been shown to increase the expression levels of several TGF- β attenuators, thus specifying the duration of the cellular response during ESC differentiation and providing an interesting mechanistic link between a hematopoietic regulator (*HOXB4*) and TGF- β signalling (Schiedlmeier et al., 2007). Our large-scale transcriptional analysis has shown that all three stromal cell lines used in this study express *TGF- β 1*, *TGF- β 2*, and *TGF- β 3* (Oostendorp et al., 2005b) as well as the *TGF- β R1*, *TGF-*

R2, and *TGF-R3* receptors and TGF- β interacting proteins such as Chordin-like (*Crim1*). The higher expression of human *TGF β 1* in the differentiating hESC cocultured with the AM20.1B4 cell line is interesting and fits well with the highest hematopoietic reconstitution achieved in the animal model used in this study. Also, the highest human *TGF β 3* was observed in hESC cocultured with stromal cells derived from the AGM and FL region, which correlates with the highest in vitro CFC activity observed in this study. These observations suggest a possible role for *TGF β 1* in hematopoietic progenitor engraftment and a likely role for *TGF β 3* in hematopoietic progenitor proliferation. It is known that Tgfb1 is produced by stromal cells, plays a critical role in proliferation and differentiation of HSCs, and at low levels is able to modulate the response of CD34+ cord blood cells to SDF1 facilitating their SDF1-mediated retention (Basu and Broxmeyer, 2005). Whether *TGF- β 1* plays a similar role in hESC differentiation to hematopoietic progenitors and their subsequent bone marrow engraftment is unknown and remains to be investigated using in vivo animal models. The role of the AGM during hematopoietic ontogeny is transient, as HSCs formed in the AGM migrate to fetal liver, which then provides the microenvironment for enhancing long-term repopulating activity and engraftment to bone marrow (Takeuchi et al., 2002). This begs the question of whether hESC-derived hematopoietic progenitors derived after exposure to the AGM microenvironment are capable of fulfilling the role of definitive HSCs. To address this question, we investigated the pattern of globin expression during hematopoietic differentiation and the capability of the hESC-derived hematopoietic precursors to differentiate along lymphoid and myeloid lineages. Our study confirmed that only embryonic and fetal globins were expressed in the erythroid colonies, suggesting the recapitulation of early hematopoiesis in our differentiation system. The lack of adult globin expression in erythroid colonies could be explained with the relatively short differentiation time used in vitro experiments (6–25 days) compared to the timing of hematopoietic development in the developing embryo. Another factor could be the lack of the correct niche at the time of hematopoietic induction/maturation, which is essential for maintaining the correct progenitor type activity. Two previous studies have indicated that hESC-derived hematopoietic cells are capable of long-term engraftment into NOD/SCID recipients without genetic manipulation (Tian et al., 2006; Wang et al., 2005). Our own studies indicated that hESC-derived hematopoietic progenitor cells, derived by coculture with the three stromal cell lines or primary cells derived from

AGM and FL region, were capable of long-term engraftment in NOD/LtSz-Scid IL2Rg null recipients. Retention of true HSC activity by hESC-derived cells was confirmed by successful secondary engraftments into the same immunocompromised recipients. Of interest was the observation that mice transplanted with hESCs differentiated in the presence of AM20.1B4 stroma showed a higher percentage of CD45+ cells in the peripheral blood when compared to animals engrafted with hESC differentiated in the presence of the UG26.1B6 stroma or AGM and FL region-derived cells as well as EL08.1D2 stromal cell line. This suggests that different inductive cues are provided by different microenvironments (niches) that need to be identified before the therapeutic potential of hESC-derived cells becomes a reality. The relatively small number of studies in this particular area makes it difficult to compare engraftment efficiencies between different groups. Notwithstanding this, the mean engraftment efficiency obtained in our study by coculture with the best cell line, AM20.1B4, is much higher (2.06%–16.26%) than the one previously reported by Tian et al. (2006) (0.16%–1.44%). The second study reported by Wang et al. (2005) does not report the mean engraftment efficiency; however, only 2 of 11 engrafted mice showed between 1% and 10% human CD45+ incorporation, making it unlikely that their engraftment efficiency would be more than 1%. Taken together, these data suggest that stromal cell lines derived from hematopoietic niches such as the AGM region provide a suitable environment for generating engraftable HSCs from hESCs. The lack of adult globin expression and definitive proof of lymphoid engraftment from hESCs do suggest that further work is needed to prove that hESCs exposed to the AGM microenvironment are capable of fulfilling the role expected for definitive HSCs.

7.5 Experimental procedures

hESC Lines

Three cell lines, H1, H9 (both from WiCell Inc.), and hES-NCL1 (derived in our laboratory) were used in this work. All data presented are an average of results obtained from the three cell lines. hESCs were grown on mitotically inactivated mouse embryonic fibroblasts with hESC medium as described in Armstrong et al. (2006).

Stromal Cell Culture

Stromal cell lines, AM20.1B4, UG26.1B6, and EL08.1D2 were maintained as described (Oostendorp et al., 2002b). The stromal cells used for hESC differentiation experiments were normally within passage 15–20. The ECM from each cell line was prepared by sodium deoxycholate method (Sigma, Dorset, UK) essentially as described by Hedman et al. (1979).

Generation of Primary Stromal Cells from the AGM and Fetal Liver

Pregnant CD1 female mice were purchased from Charles River, and embryos were dissected from the uteri at the appropriate stage. The AGM region was dissected from day 10.5 dpc embryos using fine Tungsten needles, while fetal liver was harvested from 12.5 dpc embryos. Both the AGM and fetal liver regions were subjected to a brief digestion in dispase (1.2 units/ml, Sigma) and DNase I (7 mg/ml) and then plated on gelatin coated dishes in the stromal medium described above.

Coculture of hESCs with Stromal Cells

The stromal cells were inactivated by mitomycin C treatment (10 mg/ml; 2 hr), then replated on gelatin-coated 6-well dishes at the density of 2×10^4 cells/cm². The following day, hESC colonies were placed at the top of the feeder layers (approximate density 10⁴ cells/cm²) and covered with differentiation medium containing knockout DMEM, 20% FCS (Hyclone), 10 mM β-mercaptoethanol, 1 mM L-glutamine (Invitrogen), 100 mM nonessential amino acids (Invitrogen), and 1% penicillin-streptomycin. hESCs (passage 34–45) were used for all experiments described in this manuscript. For the blocking experiments, neutralizing antibodies or chemical inhibitors were added to the differentiation media for 18 days. Control wells were incubated with differentiation medium containing a similar amount of solvent (in the case of chemical inhibitors) or isotype controls (in the case of neutralizing antibodies). For the preparation of conditioned media, the cells were mitotically inactivated as described above and plated on gelatin coated dishes at a density of 56,000 cells/cm² in stromal medium. The next day, the medium that was intended for conditioning was incubated with the mitotically inactivated stromal cells for 24 hr. The collection was repeated three times, and the pooled conditioned medium was centrifuged at 1000 rpm for 5 min to remove cellular debris.

Hematopoietic Colony Assays

All hematopoietic progenitor assays were performed according to the manufacturer's instructions (Stem Cell Technologies; Vancouver, Canada). After 12–14 days incubation at 37°C/5% CO₂ in humidified atmosphere, the plates were scored for CFU-G, CFU-M, CFU-GM, CFU-E, BFU-E, and CFU-GEMM colonies under light microscope. In each experiment, a few colonies of each type were collected by aspiration and disrupted by pipetting several times and washing in PBS. The cell suspension was cytopinned into slides (using Cytospin3, Thermo Shandon) that were subsequently fixed with methanol:acetone (9:1 volume) and then stained with Wright-Giemsa solution. The rest of the cells were stained with various antibodies for hematopoietic markers and analyzed by flow cytometry as detailed below.

Flow Cytometry Analysis

Single-cell suspensions of hESC-derived cells were prepared by incubation in cell disassociation buffer (Invitrogen) for 15–30 min at 37°C. The hESC-derived cells were washed in staining buffer (PBS+ 5% FCS) three times before staining with either fluorochrome-conjugated or unconjugated antibodies: CD45 FITC or APC, CD34 APC or FITC, CD14 FITC, CD33 PerCP-Cy 5.5, CD38 FITC, CD64 PE, CD31 FITC, CD56 FITC, CD235a PE, CD41A PerCP Cy5.5, CD105-APC (eBioscience), CD44-FITC, CD29-APC, CD71-FITC, CD54-PE, CD73-PE, CD166-PE (all from BD Biosciences, San Jose, CA), CXCR4 FITC, unconjugated KDR/VEGFR2, and SDF1 (all from R&D Systems) for 30 min at room temperature. In the case of unconjugated antibodies, secondary conjugated anti-mouse IgG/M FITC antibodies were added for another 30 min at room temperature. Appropriate corresponding IgG/M isotype controls were used in all experiments. Cells were resuspended in 0.5 ml of staining buffer and stained with propidium iodide. Live cells identified by propidium iodide exclusion were analyzed for surface marker expression using a FACS Calibur (BDIS) and Cell Quest software or FACS Aria (BDIS), using Cell Quest and Diva software.

Reverse Transcription PCR and LightCycler Quantitative

RT-PCR Analysis

Total RNA was extracted from hESC and differentiating cultures at days 6, 12, 18, 21, and 25 using Trizol reagent (Invitrogen) according to the manufacturer's

instructions. Reverse transcription and quantitative RT-PCR analysis was carried out as described in Armstrong et al. (2006). All human specific primers are shown in Table S2. Negative control samples, which lacked RT during the preparation of cDNA, were included to monitor genomic contamination. PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and visualized in a transilluminator. Analysis of NOD/LtSz-Scid IL2Rg null Mouse Engraftment

NOD/LtSz-Scid IL2Rg null cells were obtained from Jackson Labs and maintained under sterile conditions and regulations imposed by Home Office, UK. hESCs were plated on the stromal feeder cells as described above for 12 days. The whole-cell mixture was dispersed to single cells using accutase (Chemicon) for 20 min, and cells were passed through a filter to avoid debris. NOD/LtSz-Scid IL2Rg null received total body irradiation (2Sv) from an X-ray source. Twenty-four hours after the irradiation, 500,000 hESC-derived cells were injected into the left femur of each mouse. Eight to twelve weeks after transplantation, the mice were culled, and single-cell suspension from BM of each femur, spleen, and peripheral blood were collected for analysis. To prepare cells for flow cytometric analysis, red cells were lysed with 0.8% ammonium chloride solution, and the remaining cells were washed in PBS containing 5% FBS. The presence of human cells in the transplanted NOD/LtSz-Scid IL2Rg null mice was determined by flow cytometry using PerCP-Cy 5.5 conjugated antibody against human CD45 and APC-conjugated antibody against mouse CD45 (both antibodies from Becton Dickinson). In parallel, PCR analyses were performed to detect human DNA in mouse bone marrow. High-molecular-weight DNA was isolated using DNA extraction kit from Promega according to the manufacturer's instructions. PCR for the human chromosome 17-specific α -satellite was performed using forward primer 5'-ACACTCTTTTGCAGGATCTA-3' and backward primer 5'-AGCAATGTGAACTCTGGGA-3' (40 cycles, 94°C for 30 s, 60°C for 30 s, and 72°C for 15 s, followed by a final extension of 10 min at 72°C).

Statistical Analysis

A number of different statistical methods were used in this manuscript. Detailed description of each method is outlined in the specific figure legends. The results were considered significant if $p < 0.05$.

Accession numbers

The microarray data from the three stromal cell lines described in this manuscript have been deposited in the Gene Expression Omnibus under ID code GSE11589.

7.6 Acknowledgements

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7.7 Supplemental data

The Supplemental Data can also be found with this article online at <http://www.cellstemcell.com/cgi/content/full/3/1/85/DC1/>.

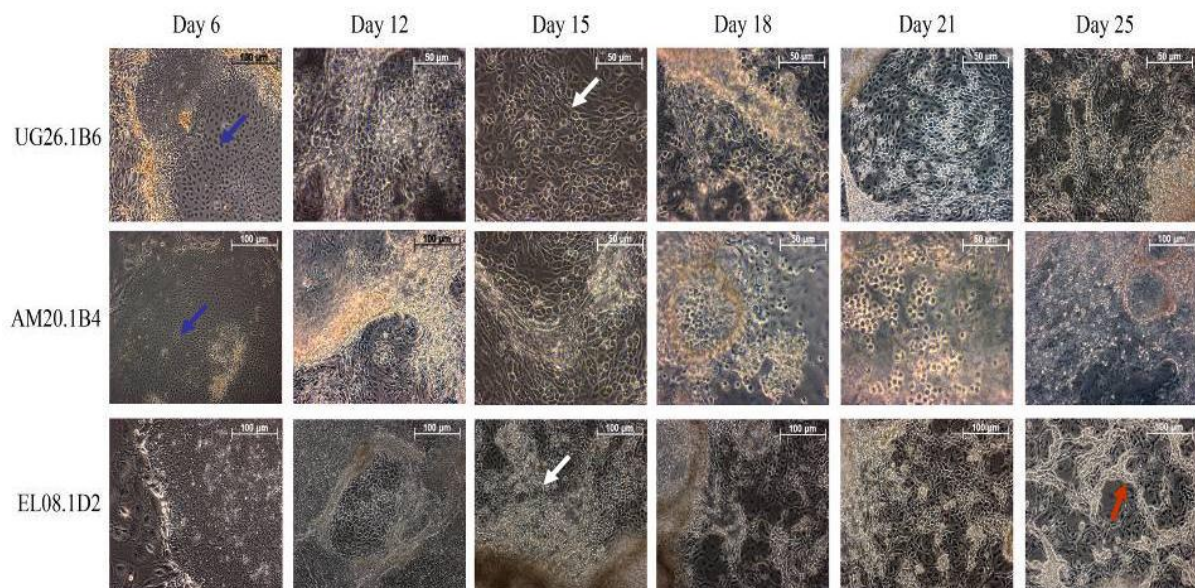


Figure S1A. Photographs of differentiating hESC on AGM (UG26.1B6, AM20.1B4) and fetal liver derived stromal cell lines (EL08.1D2). Blue arrows indicate cells with neuroepithelial like morphology, white arrows show areas with mesodermal like morphology and red arrows cells with endothelial like morphology.

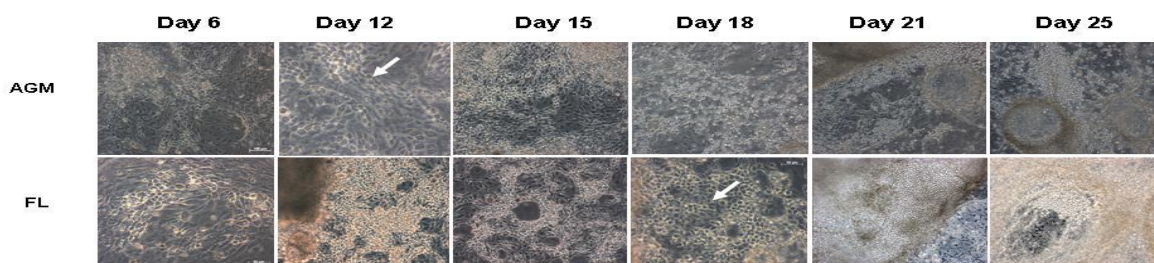
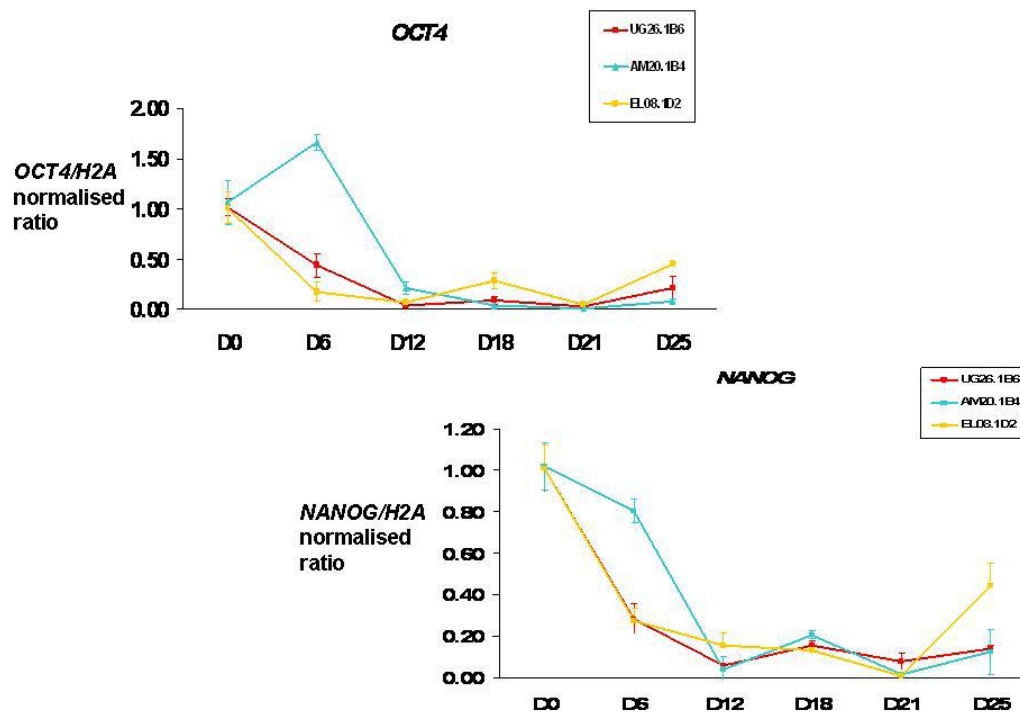


Figure S1B. Photographs of differentiating hESC on primary stromal cells derived from the AGM and FL region. White arrows show areas with mesodermal like morphology.



FigureS1C. Downregulation of *OCT4* and *NANOG* during the differentiation process as shown by quantitative RT-PCR. For each gene, the value for the undifferentiated hESC was set to 1 and all other values were calculated with respect to this (a significant downregulation of both genes is observed when day 25 expression is compared to day 0, $p < 0.05$).

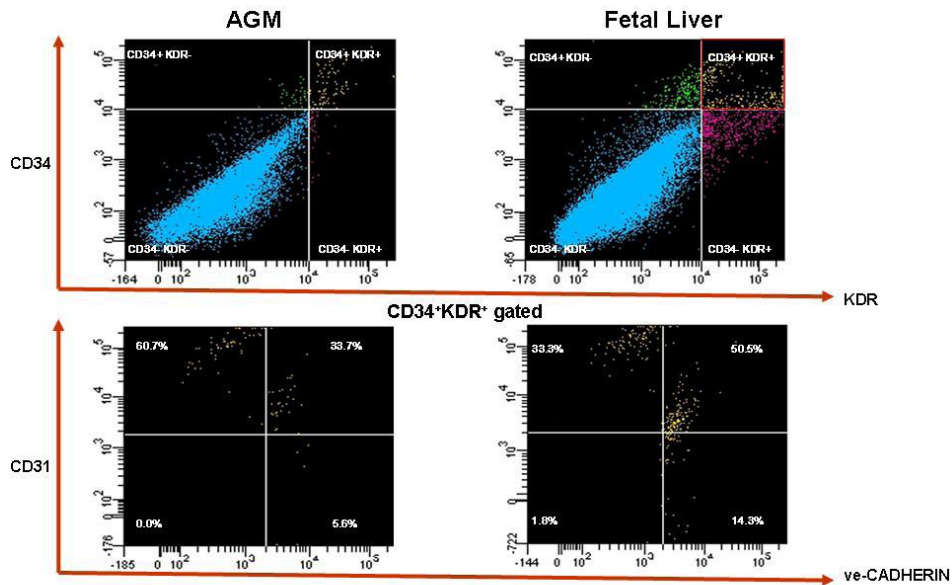


Figure S1D. Four colour flow cytometry analysis of day 18 co-cultures showing the presence of haemangioblast like cells distinguished by the co-expression of CD34, CD31, KDR and ve-CADHERIN. The expression of cells co-expressing CD34 and KDR is marked by a red gate. This gate is then examined for expression of CD31 and ve-CADHERIN which is indicated in the bottom panel for each cell line. Cells were distinguished from debris by the use of Vybrant Violet DNA tracker dye.

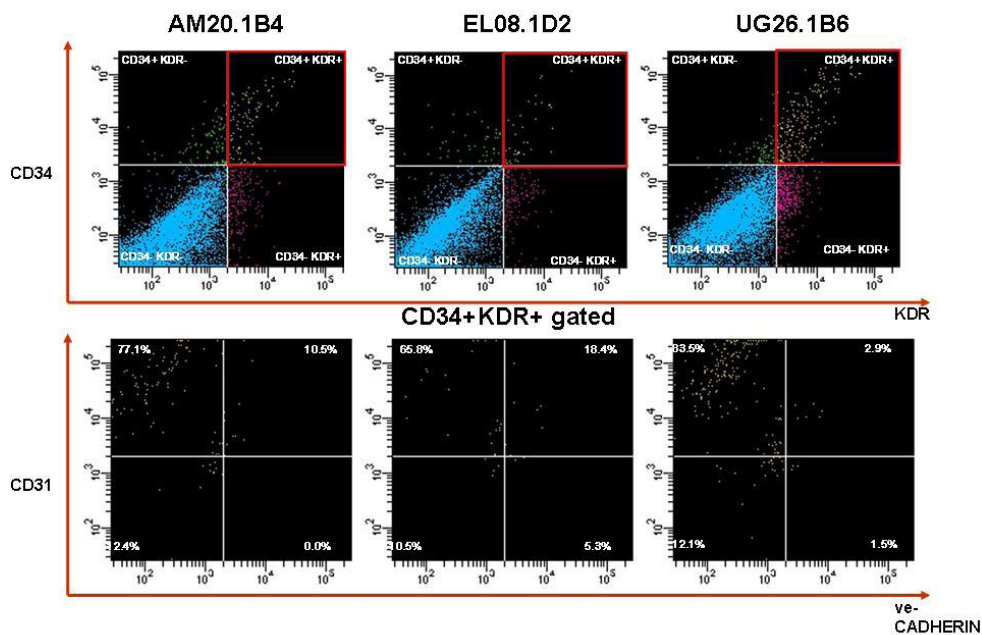


Figure S1E. Four colour flow cytometry analysis of day 18 co-cultures showing the presence of haemangioblast like cells distinguished by the co-expression of CD34, CD31, KDR and ve- CADHERIN. The expression of cells co-expressing CD34 and KDR is marked by a red gate. This gate is then examined for expression of CD31 and ve-CADHERIN which is indicated in the bottom panel for each cell line. Cells were distinguished from debris by the use of Vybrant Violet DNA tracker dye.

Granulocytes (CFU-G)

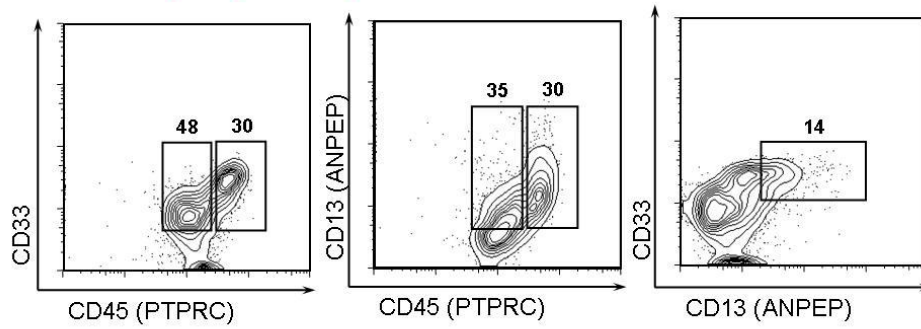


Figure S2A. Multi colour flow cytometry analysis of cells taken from CFU-G.

Macrophages (CFU-M)

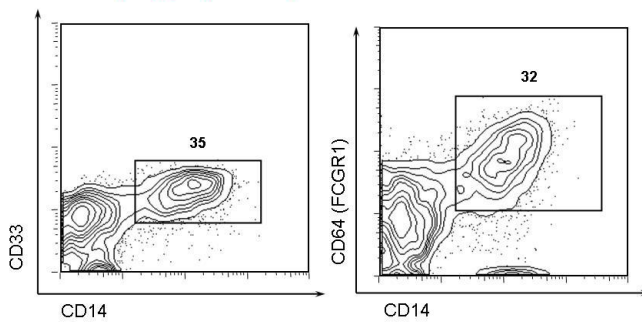


Figure S2B. Multi colour flow cytometry analysis of cells taken from CFU-M.

Mixed Granulocytes and Macrophages (CFU-GM)

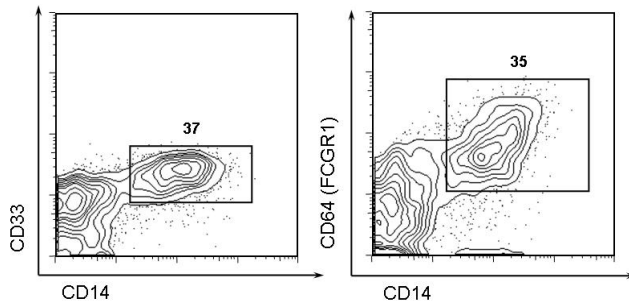


Figure S2C. Multi colour flow cytometry analysis of cells taken from CFU-GM.

Mixed Colonies (CFU-GEMM)

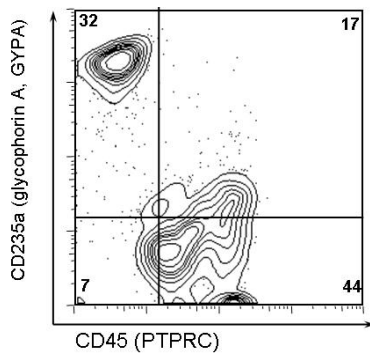


Figure S2D Multi colour flow cytometry analysis of cells taken from CFU-GEMM colonies obtained from the differentiation of hESC on immortalised stromal cell lines. The presence of erythroid cells within these colonies is indicated by the expression of CD235A and lack of CD45.

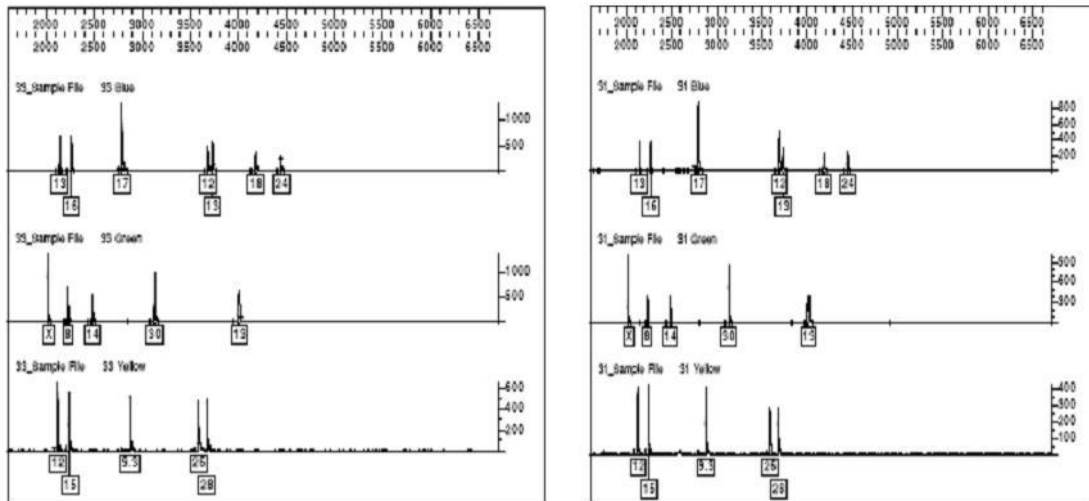


Figure S2E. Fingerprinting analysis of H9 human ESC (left panel) and cells taken from CFC colonies taken after the H9 co-culture with the fetal liver primary cells (right panel). Microsatellite comparison reveals identical profile indicative of a common genetic origin.

AM20.1B4

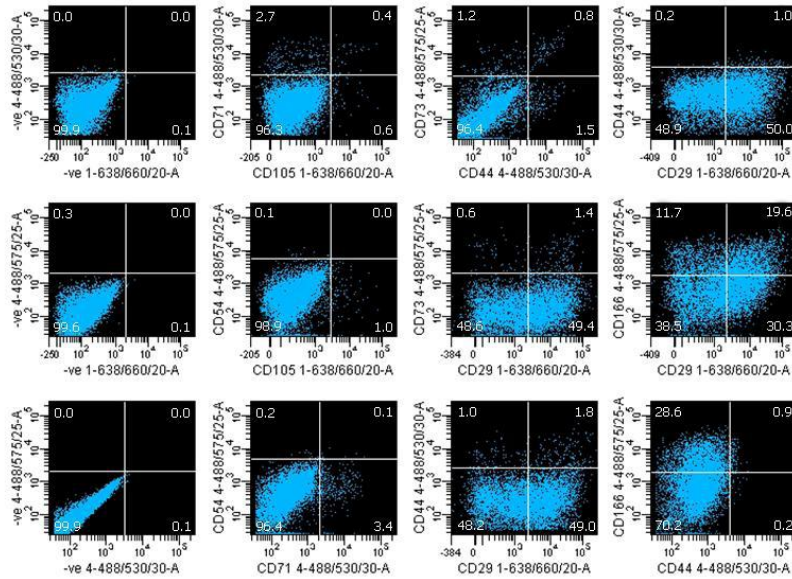


Figure S3A. Five colour flow cytometry analysis was carried out to determine presence of MSC like cells using a combination of antibodies found in MSCs (CD29, CD73, CD44, CD54, CD166, CD71 and CD106) in hESC differentiated cells lacking CD34 and CD45 expression. Cells were distinguished from debris by the use of Vybrant Violet DNA tracker dye.

EL08.1D2

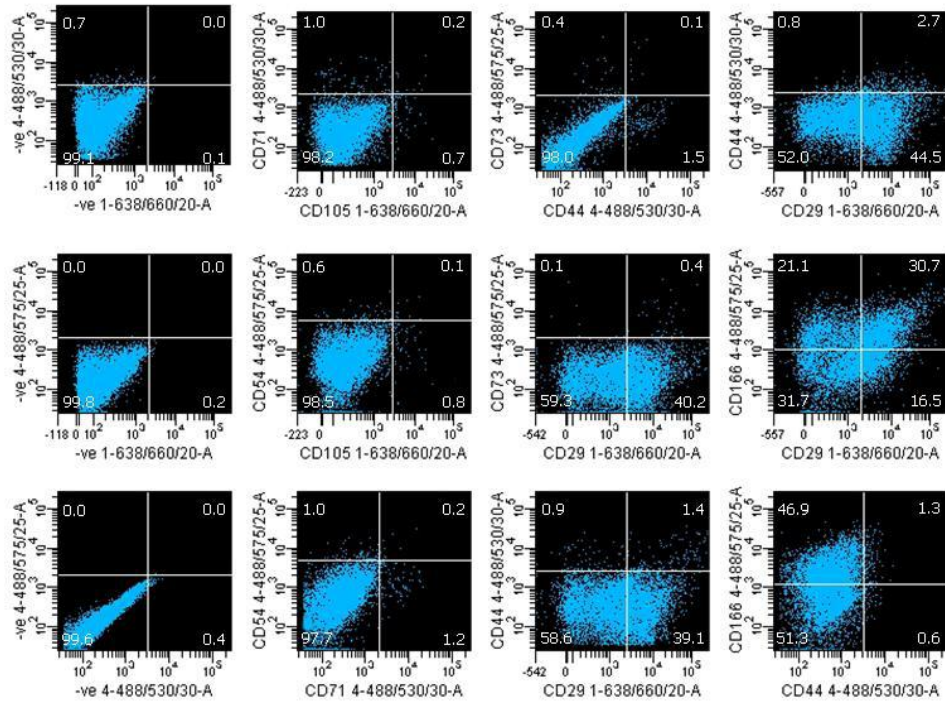


Figure S3B. Five colour flow cytometry analysis was carried out to determine presence of MSC like cells using a combination of antibodies found in MSCs (CD29, CD73, CD44, CD54, CD166, CD71 and CD106) in hESC differentiated cells lacking CD34 and CD45 expression. Cells were distinguished from debris by the use of Vybrant Violet DNA tracker dye.

UG26.1B6

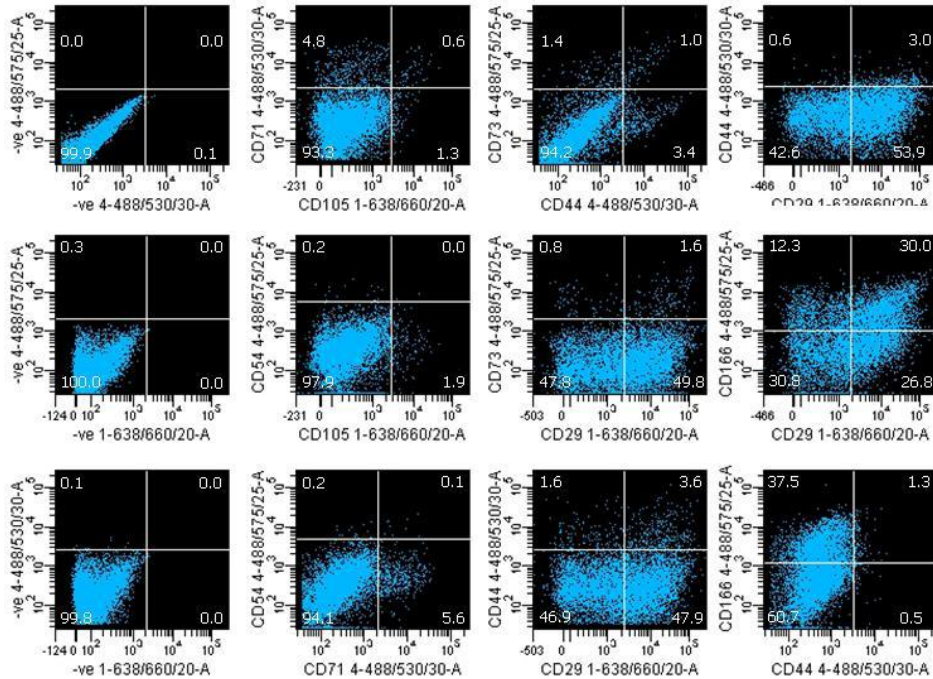


Figure S3C. Five colour flow cytometry analysis was carried out to determine presence of MSC like cells using a combination of antibodies found in MSCs (CD29, CD73, CD44, CD54, CD166, CD71 and CD106) in hESC differentiated cells lacking CD34 and CD45 expression. Cells were distinguished from debris by the use of Vybrant Violet DNA tracker dye.

Figure S4

The microarray data from the three stromal cell lines described in this manuscript have been deposited in the "Gene Expression Omnibus" with the following Accession numbers:

Accession	Title
GSE11589	Gene expression analysis of embryo-derived stromal cell lines
GSM292046	Irradiated EL08-1D2, biological rep 1
GSM292047	Irradiated EL08-1D2, biological rep 2
GSM292048	Irradiated UG26-1B6, biological rep 1
GSM292049	Irradiated UG26-1B6, biological rep 2
GSM292050	Irradiated AM20-1B4

Inhibitor Name	Function	Conc.	Specificity	Source	CD45 (expressed as % of control)
ALK 4-5-7 (Chemical Inhibitor)	Inhibitor of Activin receptor 1 (ALK4), TGFβ receptor 1 and Nodal receptor 1 (ALK7), implicated in the TGFβ superfamily signalling pathways	10μM	Human/ Mouse	Sigma	7
PLCy (Chemical Inhibitor)	Phospholipase C α subunit: a multidomain phosphodiesterase involved in cleavage of polar head groups from inositol lipids, implicated in calcium signalling pathways (PPI --> IP3 inhibitor, leads to decrease in cytosolic Ca+)	10μM	Human/ Mouse	Sigma	15
FGF7 (Blocking Antibody)	Fibroblast growth factor 7: a potent epithelial cell specific growth factor, whose mitogenic activity is predominantly exhibited in keratinocytes but not fibroblasts or endothelial cells. Mouse and rat homologs are implicated in morphogenesis of epithelium, reepithelialisation of wounds, hair development and early lung organogenesis.	25μg/ ml	Human R&D	Systems	40
FGF2 (Blocking Antibody)	Basic fibroblast growth factor (bFGF or FGF2), is a multifunctional growth factor involved in cell growth and in tumor development and progression, and is also a mitogen with prominent angiogenic properties. bFGF binds to all four FGF receptors, which induces receptor dimerisation and autophosphorylation, allowing binding and activation of downstream targets including FRS2, PLC and Crk.	2.5μg/ ml	Mouse	AbCam	41
Cathepsin K (Chemical Inhibitor)	Cathepsin K expression is stimulated by inflammatory cytokines, and is able to catabolise elastin, collagen, and gelatin to function in bone and cartilage resorption	50nM	Human/ Mouse	Calbioc hem	47

FGF9 (Blocking Antibody)	Fibroblast growth factor 9 is a secreted factor that exhibits a growth-stimulating effect on cultured glial cells. Murine FGF9 expression is dependant on Sonic hedgehog (Shh) signaling and male-to-female sex reversal is observed in its absence, suggesting a role in testicular embryogenesis.	5µg/ml	Mouse	AbCam	48
Eotaxin (Blocking Antibody)	Eotaxin, a chemokine ligand for the CCR3 receptor, recruits and activates eosinophils. CCR3 activates multiple signalling pathways including PI3Ky, Rho/ROCK and PLCβ to stimulate inflammatory responses and intracellular calcium release resulting in changes in stress fiber formation via pertussis sensitive actin polymerisation.	20µg/ml	Human/ Mouse	R&D Systems	50
TGFβ3 (Blocking Antibody)	Transforming growth factor beta 3 is a multifunctional cytokine involved in proliferation, growth, differentiation, motility, and in deposition of extracellular matrix. Proteolytic processing of latent inactive TGFβ complex is required for its activation, mediated through the TGF receptors I and II, and stimulates the phosphorylation of Smads 2 and 3.	1µg/ml	Human/ Mouse	MBL	53
IFNγ (Blocking Antibody)	Interferon gamma binds the IFNγ receptor to mediate activation of the JAK/STAT signalling pathways	5µg/ml	Mouse	R&D Systems	55
TGFβ1 - SB203580 (Chemical Inhibitor)	A potent inhibitor of TGFβ1 signalling and p38 MAP kinase. TGFβ1 is the most abundant of the TGFβ signalling factors and is produced by almost all cell types.	100nM	Human/ Mouse	Calbiochem	57
IGFBP3 (Blocking Antibody)	Insulin-like growth factor binding protein 3 is implicated in prolonging the half-life of insulin growth factors (IGFs) and altering their interaction with cell surface receptors to signal through the IGF-receptor I pathway	5µg/ml	Human	R&D Systems	58
CCL2 (Blocking Antibody)	Chemokine (C-C motif) ligand 2 binds to chemokine receptors CCR2 and CCR4. It displays chemotactic activity for monocytes and basophils and has been implicated in diseases characterised by monocytic infiltrates (e.g. psoriasis, rheumatoid arthritis and atherosclerosis). Additionally CCL2 is a downstream member of the VEGF/VEGFR2 signalling pathway and may be involved in cell proliferation via NFκβ and AP-1.	5µg/ml	Mouse	R&D Systems	64

IL6 (Blocking Antibody)	Interleukin 6 stimulates the IL-6 receptor signalling pathway to directly activate JAK1 signalling whilst cleaving ADAM 10 and 17 proteins. IL-6 binding can also indirectly activate the gp130/STAT3 signalling pathway	5µg/ml	Mouse	R&D Systems	66
M-CSF (Blocking Antibody)	Macrophage colony-stimulating factor (M-CSF) is critical for osteoblast development and function, although its nuclear targets in osteoclasts are largely unknown	5µg/ml	Mouse	R&D Systems	66
SDF1 (Blocking Antibody)	Stromal cell derived factor 1 (SDF1, CXCL12) is a small, pro-inflammatory chemoattractant cytokine that regulates leukocyte trafficking by interacting with G protein-coupled receptors. Many embryonic and somatic stem or progenitor cells express the SDF1 receptor CXCR4 and their homing and maintenance regulated by SDF1.	50µg/ml	Human/ Mouse	R&D Systems	66
IGFBP6 (Blocking Antibody)	Insulin-like growth factor binding protein 6 signals through the IGF-receptor I pathway and is additionally regulated by β -catenin and may be a target of the Wnt signalling pathway	20µg/ml	Human	R&D Systems	70
IL1-R1 (Blocking Antibody)	Interleukin 1 receptor type I, implicated in the NF κ B, and IL1/IL-6 signalling pathways	5µg/ml	Mouse	R&D Systems	75
FGF-R1 - SU5402 (Chemical Inhibitor)	Inhibits the tyrosine kinase activity of fibroblast growth factor 1 (FGF-R1), and inhibits the aFGF-induced phosphorylation of ERK1 and ERK2.	10µM	Human/ Mouse	Calbiochem	76
TNFα (Blocking Antibody)	Tumor necrosis factor alpha is a multifunctional pro-inflammatory cytokine mainly secreted by macrophages. It functions by binding its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFR2, and is involved in the regulation of cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation.	25µg/ml	Mouse	R&D Systems	76
IL-3 (Blocking Antibody)	Interleukin 3 promotes haematopoietic stem cell proliferation and differentiation through binding to IL-3 receptor heterodimers and signalling primarily through the Stat5 and MAPK pathways	2µg/ml	Mouse	R&D Systems	79

TGFβ 1+2 (Blocking Antibody)	The transforming growth factor beta superfamily consist of multifunctional cytokines involved in diverse cell functions including proliferation, growth, differentiation, motility, and deposition of extracellular matrix. Proteolytic processing of the latent inactive TGFβ complex is required for its activation, mediated through the TGF receptors I and II and stimulates the phosphorylation of Smad 2 and 3.	20µg/ml	Human/ mouse	R&D Systems	79
VEGFR2 (Blocking Antibody)	Vascular endothelial growth factor receptor 2 (VEGFR2, KDR, Flk-1) transduces VEGF induced signalling, proliferation, chemotaxis and sprouting in endothelial cells <i>in vitro</i> , as well as angiogenesis <i>in vivo</i> . VEGFR2 activation leads to recruitment of adaptor proteins, including Shc, GRB2, PI-3 kinase, Nck, SHP-1 and SHP-2, leading to binding of Grb2, PI-3 kinase, PLCγ and Shb.	20µg/ml	Mouse	R&D Systems	80
GAS6 (Blocking Antibody)	N-acetylgalactosamine-6-sulfatase is a lysosomal exohydrolase that acts through Axl/Fc receptors and is required for degradation of glycosaminoglycans, keratan sulfate, and chondroitin 6-sulfate, mediated through the mTOR/Akt/PI3K signalling pathways. It is also thought to be important in PGC growth and sexual differentiation.	50µg/ ml	Human	R&D Systems	82
TPO (Blocking Antibody)	Thrombopoietin is a membrane-bound glycoprotein that acts as an enzyme and plays a central role in thyroid gland function. TPO receptor signalling initiates aggregation and activation via several major pathways including MAPK (ERK and JNK), Protein Kinase C, and JAK/Stat.	5µg/ml	Human/ Mouse	R&D Systems	87
CCL5 (Blocking Antibody)	Chemokine (C-C motif) ligand 5 functions as a chemoattractant for blood monocytes, memory T helper cells and eosinophils and causes the release of histamine from basophils. Signalling is primarily through the ERK/AKT pathways at low ligand levels, and Src/MEK/ERK at high ligand levels.	1µg/ml	Human/ Mouse	R&D Systems	90
SCF (Blocking Antibody)	Stem cell factor is the ligand of the tyrosine-kinase receptor encoded by the KIT locus. This ligand is a pleiotropic factor that acts in utero in germ, neural and haematopoietic cell development, reflecting a role in cell migration	5µg/ml	Mouse	R&D Systems	94

IGFBP4 (Blocking Antibody)	Insulin-like growth factor binding protein 4 binds both insulin-like growth factors (IGFs) I and II, which circulates in the plasma, prolongs the IGF half-life, and alters signalling through the IGF-receptor I pathway	50µg/ml	Human	R&D Systems	95
GM-CSF (Blocking Antibody)	Granulocyte macrophage colony stimulating factor is a cytokine that controls production, differentiation and function of granulocytes and macrophages. The active form of the protein is found extracellularly as a homodimer, but little is known about GM-CSF targets or signalling pathways.	2µg/ml	Mouse	R&D Systems	107
IGF2 (Blocking Antibody)	Insulin-like growth factor 2 is an imprinted gene expressed only from the paternally inherited allele and is involved in development, growth and proliferation.	5µg/ml	Mouse	R&D Systems	111
VEGF-D (Blocking Antibody)	Vascular endothelial growth factor binds to VEGF receptors I, II and III, and can induce KDR and PLCγ tyrosine phosphorylation. VEGF-D is strongly implicated in PI3K mediated Akt activation leading to PI3K-dependent endothelial nitric-oxide synthase (e-NOS) phosphorylation.	5µg/ml	Human+ 25% Mouse	R&D Systems	114
TGFβ R III (Betaglycan)	This type III receptor is a transmembrane proteoglycan binds TGFβ2 with highest affinity	0.5-6 ng/ml	Human	R&D Systems	152
BMP2+BMP4 (Gremlin Blocking Antibody)	A secreted glycoprotein that binds and antagonises bone morphogenic protein (BMP) 2 and 4	1ng/ml	Mouse	R&D Systems	155
Carboxypeptidase M and N (Plummer's Chemical Inhibitor)	A potent inhibitor of human carboxypeptidase M and N, and bradykinin hydrolysis. Carboxypeptidase enzymes hydrolyse C-terminal peptide bonds, and are involved in blood clotting, growth factor production, wound healing and reproduction, where they are involved in protein maturation.	50µM	Human/ Mouse	Calbiochem	167
ALK 4 (Blocking Antibody)	ALK4 is the membrane bound Activin receptor type IB. In Xenopus, treatment with ALK4 is known to produce similar results to Activin treatment, including dorsoanterior mesoderm and endoderm specification.	1µg/ml	Human	R&D Systems	175
BMP4 (Blocking antibody)	Bone morphogenic protein 4, a member TGFβ superfamily, is secreted into the extracellular matrix. It is primarily involved in initiation and promotion of bone development and regeneration, and signals through the TCF/β-Catenin or Smad 1/5/8/Erk pathways.	2.5µg/ml	Human/ Mouse	AbCam	210

Table S1. Candidate signalling pathways were selected from large scale transcriptional studies carried out in murine UG26.1B6 stromal cell line. Inhibition was achieved by using neutralising antibodies or known chemical inhibitors. The effects on haematopoietic differentiation were assessed by changes in CD45 expression at day 18 of differentiation. The data represents the average of three independent experiments.

Product	Forward primer Sequence	Reverse primer Sequence	Optimal temperature	Size (bp)
CD34	GAGGCCTCAGTGTCTACTGC	CGATTCATCAGGAAATAGCC	61	228
KDR/VEGFR2	CAGACGGACAGTGGTATGGT	AGCCCAGATTCTCCAGCCTG	55-64	268
PU.1	CACAGCGAGTTCGAGAGCTT	GATGGGTA CTGGAGGCACAT	61	194
SCL	AGTCATGAATGCACCAGAAC	CACAACCTGTCTGCTCACTCGA	54-55	353
LMO2	GGATCTGCGCGAGAGACTAT	GAATTCAGTGAACCTCCGCAAA	53-55	289
BRACHYURY (T Protein)	CCTATTCTGACAACCTCACCTG	CTGCGGCGTCTACTGGCTGT	57	351
RUNX1a/AML1a	AAGACACAGCACCTGGAGA	GCCTCCCTCATAACGTGCAT	60	151
RUNX1b/AML1b	CGTGCACATACATTAGTAGCA	CTTCCACGAATCTTGCTTG CAGAGGTTAAG	60	304
RUNX1c/AML1c	GAAGTCTGAACCCAGCATAGT	GTGGACGTCTCTAGAAGGATTCATTCCAAG	60	231
GATA 2	CAAGTACCAGGTGCTACTGA	GTGCATGCAGGAGAAGTCAT	54-55	517
FLT3	GGACGGACATAAGGTGCTGT	GTGTCGTTTCTTGCCACTGA	53	298
CDX4	CTGTGGGCGGTGGA ACTA	AAACTACACGATACTTTCTTTGTCC	61	186
HOXB4	GTCGTCTACCCTGGATGC	CGTGT CAGGTAGCGGTTGTA	59	153
MIXL	AGCTGCTGGAGCTCGTCTT	AGAGAGGGGAACAGGTTTCAA	53	336
EKLF	CGGACACACAGGATGACTTC	GGCTGGTCTCAGACTTAC	59-60	115
δ-Globin (HBD)	TCCTGAGGAGAAGACTGCTGT	GAGAGGACAGATCCCCAAAGGACTCAAA	62	268
ξ-Globin (HBAZ)	ACCAAGGCCAGTCTGAG	CGGGTGGCTGAGGAAGAG	53	169
ε-Globin (HBE1)	ATATCTGCTCCGACACAGC	GCTTGAGGTTGTCCATGTTT	53	303
α-Globin (HBA1)	ACTTCTGGTCCCACAG	GTGGGAAGGACAGGAAC	59	153
β-Globin (HBB)	GCAACCTCAAACAGACACCA	TTAGGGTTGCCATAACAGC	55	202
γ-Globin (HBG1)	CACAGAGGAGGACAAGGCTA	CTTGAGATCATCCAGGTGCT	63	238
CD31	GCTGACCCTTCTGCTCTGTT	TGAGAGGTGGTGTGACATC	55	238
vWF	CCCGAAAGGCCAGGTGTA	AGCAAGCTTCCGGGGACT	61-63	228
SDF1α	TCAGCCTGAGCTACAGATGC	CTTAGCTTCGGGTCAATGC	57	165
LIFR	TGCCATTCA TTTTG TGAAA	GGATCAAGGGG CAGTTTGTA	52	237
LIF	CTGTTGTTCTGCACTGGAA	GCCACATAGCTTGTCCAGGT	55	220
C-Met	TGAAATGCACAGTTGGTCTT	AGCCATAGGACCGTATTTTCG	55	150
NANOG	AGAAGGCCTCAGCACCTAC	GGCCTGATTGTTCCAGGATT	57	205
OCT4	TCTCGCCCCCTCCAGGT	GCCCCACTCCAACCTGG	53	218
HUMAN CHROMOSOME 17 α-SATELLITE#	ACACTCTTTTTGCAGGATCTA	AGCAATGTGAAACTCTGGGA	60	1171
HUMAN TGFβ1	ACATTGACTCCGCAAGGAC	CCGGTTATGCTGGTTGTA	62	150
HUMAN TGFβ3	AGTGCAGACACAACCCACAG	CGTGGGGTCTCAGCTACATT	62	192
MOUSE Tgf1	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG	65	133
MOUSE Tgf3	CCTGGCCCTGCTGAACTTG	TTGATGTGGCCGAAGTCCAAC	62	75
H2A	GTCGTGGCAAGCAAGGAG	GATCTCGGCGGT CAGATAC	60	181
HUMAN GAPDH	GTCAGTGGTGGACCTGACCT	CACCACCCTGTTGCTGTAGC	53-62	268
MOUSE GAPDH	TGGTGAAGGTCGGTGTGAAC	CGTTGAATTTGCCGTGAGTG	62	165

Table S2. Oligonucleotides used in the quantitative RT-PCR analysis. The numbers shown in superscript indicate the references from where the oligonucleotide sequences were obtained.

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Chapter 8: Conclusion and future aspects

8.1 Conclusion

At the start of this project we faced a multitude of challenges; first of all we didn't have that much to go on when looking for possible candidates that could influence hematopoiesis in an extrinsic manner; secondly, there were technical challenges requiring setting up new technologies, such as the yeast 2 hybrid and proper staining protocols for more advanced and comprehensive hematopoietic FACS stains.

During the course of the project, it became clear that the intended identification of new candidate factors for extrinsic regulation could not be found using the yeast two-hybrid technology. This problem was overcome with new and more extensive gene expression arrays, establishment and optimisation of methodologies that now are standard in the lab. The gene expression analysis paved the way for two publications in the journal "Cell Stem Cell", and a submitted review to Chemical-Biological Interactions, which I might add was worth the effort.

The study described in this thesis, shows that stromal cell lines which support hematopoiesis are useful tools to find mediators of hematopoiesis by comparing them to non-supportive cell lines. The previous study investigating the cell lines supportive potential did identify *Sfrp2* as a possible candidate to regulate hematopoiesis (Oostendorp et al., 2005), we can now show that a related gene, *Sfrp1*, is truly such a regulator (Renstrom et al., 2009) and that UG26-1B6 and AM20-1B4 can induce the formation of hematopoietic progenitors, a process in which *TGFb1* and *TGFb3* could all have their roles to play (Ledran et al., 2008).

8.2 Human embryonic stem cell project

In collaboration with Lako and co-workers we helped to investigate if the UG26-1B6, EL08-1D2 and AM20-1B4 cell lines have the potential to force the differentiation of human embryonic stem cells (hESC) into hematopoietic progenitor cells. As it turns out the UG26-1B6 had the best results in vitro studies while the AM20-1B4 co-culture led to the best results in vivo, which was measured by the amount of human donor cells found in the circulation of immunologically compromised mice. It was also revealed that there were differences in how these three cell lines affected the hESC, the AM20-1A4 seemed to act more in the way of inducing differentiation and thus giving more primitive precursor cells while UG26-1B6 could act by expanding the already existing pool of progenitors, subtle as it might be it shows that there are differences in how the cell lines support hematopoiesis. Our gene expression

analysis revealed that *TGFb1*, *TGFb2* and *TGFb3*, were all slightly higher expressed in AM20-1B4 in comparison to EL08-1D2 and UG26-1B6. Subsequently, the Lako group demonstrated that all three members of the TGF family affect the differentiation of hESC towards the hematopoietic lineage as well as their subsequent hematopoietic activity. The exact role of these mediators in vivo engraftment was not explored. But, since it has been described that *TGFb1* could affect the *SDF1* mediated retention CD34+ cord blood cells (Takeuchi et al., 2002), and *TGFb3* the proliferation of hematopoietic progenitors, one could speculate that these mediators probably do affect engraftment. In the end it showed that using stromal cell lines could be a possible way to produce hematopoietic stem- or progenitor cells which could then be used for therapeutical purposes.

8.3 Sfrp1 as a modulator of hematopoiesis

Our gene expression analysis revealed that *Sfrp1* was overrepresented in the two cell lines which support adult HSC in culture. By investigating mice deficient in *Sfrp1* we found that its loss resulted in an increased number of peripheral blood cells, mostly affecting lymphoid B220+ cells and myeloid Gr1+ cells. The alteration to the amount of B220+ cells was in line with findings of (Yokota et al., 2008), which were published in the course of our investigations. At the moment, we cannot explain the increase in blood cell numbers. Since mice having a stabilized form of *Ctnnb1* suffer from acute depletion of blood cell numbers, (Kirstetter et al., 2006; Scheller et al., 2006), one could hypothesize that mice with a reverse phenotype would show increased blood cell numbers. Indeed, the loss of *Sfrp1* leads to decreased *Ctnnb1* levels in HSC and MPP. However, whether *Ctnnb1* was also decreased in more mature blood cells, we did not investigate in this study.

Yet, we show that *Ctnnb1* levels were symptomatic for our observed phenotype. Also, our findings are in line with the previous report from Scheller et al., who state that mice with stabilized *Ctnnb1* retain less LSK cells in the G0/G1 phase of the cell cycle. Considering that *Sfrp1*^{-/-} mice exhibit the reverse, namely less *Ctnnb1*, with more LSK cells in this “resting” phase, our data challenge the view that *Sfrp1* is an inhibitor of canonical Wnt signalling. Instead, we suggest that that *Sfrp1* may have hitherto unrecognized agonistic Wnt signalling activity.

The exact mechanism by which the *Ctnnb1* levels decrease in the absence of *Sfrp1* are unclear at present. Our collaborators recently showed that *Sfrp1* binds to Wnt5a,

the most important stimulator of non-canonical Wnt-signalling (Matsuyama et al., 2009), and the loss of *Sfrp1* would then remove some of the restraints on *Wnt5a* resulting in an increased degradation of *Ctnnb1* (Topol et al., 2003). To support such a hypothesis we also found that, *Pparg*, was over-expressed in many, but not all, immature cells (MPPs) lacking *Sfrp1*. *Pparg* is known to suppress *Ctnnb1* levels in colon cancer (Girnun et al., 2002) and also in adipogenesis (Moldes et al., 2003), possibly through a proteasome dependent mechanism (Sharma et al., 2004). Another possibility is that *Sfrp1* directly regulates proteasomal regulation of *Ctnnb1*. Although such a mechanism has not been explored in the literature we cannot exclude that *Sfrp1* (or lack of it) regulates components of the *Ctnnb1* destruction complex (axin, APC, GSK3b), or directly affects the SCF-TrCP ubiquitin ligase involved in proteasomal marking *Ctnnb1*.

Ctnnb1 directs a transcriptional machinery to regulate what are called “classical” canonical Wnt targets. We show that one important target of the canonical pathway, *Ccnd1*, was down-regulated transcriptionally in Cd34- LSK cells. The decreased *Ccnd1* levels are in line with our other finding that primitive hematopoietic cells seem to favour retention in the G0/G1 phase of the cell cycle. Interestingly, we can also show that the more mature cells differ here compared to the more immature ones by being less in G0/G1 than their control counterparts, suggesting that the Wnt signalling influences the cell cycle status of more mature cell types in a different way compared to immature cells.

In the study of *Sfrp1*^{-/-} mice, we focused on the analysis of early hematopoietic subsets. Thus, we cannot explain why the mature cells are cycling more than the controls. Here, it seems that if *Pparg*, or a possibly altered non-canonical signalling, that are responsible for the alterations in primitive cells, may not affect signalling changes in mature cells. Again, future work should determine the role of *Pparg* and non-canonical signalling in the regulation of mature cell subsets. Having said that, when it comes to the regulation of the mature hematopoietic cells cell cycle, the *Sfrp1*^{-/-} mice show similarities to what has been described for *Wnt5a*^{+/-} mice which also show increased peripheral blood cell numbers and B cells (Liang et al., 2003). This however, would actually suggest that *Wnt5a* and *Sfrp1* share functions in mature hematopoietic cells. In addition, if one compares the *Wnt5a*^{-/-} with the *Sfrp1*^{-/-}*Sfrp2*^{-/-} mice, who are not viable and die around day E16.5 (*Sfrp1*^{-/-}*Sfrp2*^{-/-}) and E18.5 (*Wnt5a*^{-/-}), further phenotypical similarities with somite alterations, shortened snout

resulting in an almost identical cranial features and deformed shortened limbs are apparent (Sato et al., 2006; Yamaguchi et al., 1999).

The comparison made with the *Sfrp1^{-/-}Sfrp2^{-/-}* mice is due to the fact that *Sfrp1* and *Sfrp2* are reported to be functionally redundant (Sato et al., 2006). That could mean that perhaps the two *Sfrp* genes are together functionally similar to *Wnt5a*, but at the same time *Sfrps* might also be needed for the *Wnt5a* to function, at least to a high degree, which would explain why those knockouts have such overlapping phenotypes. The above hypothesis, that *Sfrp1* is primarily binding to *Wnt5a*, inhibiting it, and a knock out of *Sfrp1* would lead to a *Wnt5a*-mediated degradation of *Ctnnb1*, is rather speculative but could also hold some merit. This especially since if *Sfrp1* itself would be an antagonist of canonical signalling it could explain the findings by Uren et al. (Uren et al., 2000), why *Sfrp1* could act as an agonist at lower concentrations. In order to prove this, future studies should resolve whether the specificity of *Sfrp1* is higher to *Wnt5a* than to other *Wnts*, or that *Sfrp1* affects *Wnt5a* binding to its *Fzd* receptors. Such experiments could shed light on the observed similarities between the *Wnt5a^{-/-}* and *Sfrp1^{-/-}Sfrp2^{-/-}* in which both would be the result of an inhibitor of canonical *Wnt* signalling “running loose” without any inhibition. To clarify; *Sfrp1/2* inhibiting without the hindrance of *Wnt5a* in the *Wnt5a^{-/-}* mouse and then *Wnt5a* doing the same in the *Sfrp1^{-/-}Sfrp2^{-/-}*. But still the exact interactions between *Wnt5a* and *Sfrp1* are still out of reach and up for debate and will need further study.

Several studies have shown that different signalling pathways interact to regulate self-renewal of HSC. Indeed, we also found changes to the other genes that could be due to non-*Wnt* related pathways dependent on *Sfrp1*. *Hes1*, a direct target of the Notch signalling pathway and known inducer of quiescence (Yu et al., 2006), was one of these genes and fits well with the picture that emerged from the *Sfrp1^{-/-}* HSCs which were retained in the G0/G1 phase since *Hes1* was found to be up regulated. Together with our finding that *Ccnd1* was down regulated, one could propose that *Ccnd1* might regulate *Hes1*, a hypothesis planned to be tested in a follow-up study. In addition to *Hes1*, we further found that two genes involved in lineage specification and development, *Runx1* and *Ikbkg*, were differentially expressed in *Sfrp1^{-/-}* which also fits with the altered ratios of mature cells that we found. Something which was not suggested before was that *Runx1* could be directly or indirectly regulated by members of the *Wnt* signalling pathway and the down regulation of *Runx1* in LSK

and the up regulation in MPP suggests that it might be involved in the machinery separating self-renewing LSK from differentiating MPP.

In short we can here clearly show that *Sfrp1* is an important regulator of hematopoiesis mainly by regulating the cell cycle and perhaps also differentiation commitment of hematopoietic cells. Even though the exact mechanism is still not full known it seems likely that altering to the canonical and/or non-canonical Wnt pathway is the most probable reason.

8.4 Future aspects

Recent findings have shown the emergence of stroma derived factors as very important in regulating hematopoiesis. One of these has turned out to be *Sfrp1* which evolved to be the main focus of my thesis. Even though we have explored much of the actions of *Sfrp1* in hematopoiesis, there are many unresolved issues that need to be further explored in order to fully understand how *Sfrp1* regulates hematopoiesis. Several questions for the future can be formulated.

For instance, although it is clear that balance between canonical and non-canonical Wnt-signalling is important for HSC self-renewal, exactly how *Sfrp1* and the non-canonical Wnt ligand *Wnt5a* interacts has not been resolved. The question remains what, indeed, is the role of *Wnt5a* in the changes in hematopoietic regulation seen in *Sfrp1*^{-/-} mice? Another unresolved issue is the relationship between *Sfrp1* and its partially redundant family member *Sfrp2*. Why is the loss of *Sfrp1* not compensated by the presence of *Sfrp2*? Also, since it is known that *Sfrp1* is down-regulated in many types of cancer, the question emerges whether the lack of *Sfrp1* will facilitate the development of leukemia? Indeed, my preliminary work (not in this thesis), suggests that old *Sfrp1*^{-/-} do indeed develop hematopoietic malignancies of both lymphatic and myeloid lineages. Thus, the above questions may all be tied into possible future clinical applications of *Sfrp1* or *Sfrp1* agonists which are the main goal of basic research.

Our discovery of *TGFb1* and *TGFb3* as enhancers of hematopoietic differentiation for hESCs will have similar potential as *Sfrp1*. Perhaps these studies will lead to development of “specialised” stroma cultures, or serum-free media based on components of stromal cells. Ever since the description of induced pluripotent cells (iPS), many investigators developed techniques that utilise the patients own cells, to produce cell needed to treat certain maladies. Thus, medical science is closing in on individually tailored treatments will see more and more use in the future as the technology continue to improve and enable such treatments to be practically feasible on a larger scale. In this regard, it will be of interest to see whether iPS can be directed towards the development of definitive HSC in a similar way as hESC, and whether the TGFB family members play a similar important role in promoting hematopoietic development.

In conclusion, the use stroma cell lines like the ones that were used in this thesis has led to potential new factors that extrinsically regulate hematopoietic development and

maintenance of HSC. It is my hope that future work will lead to clinical applications of these factors or of the mechanisms underlying their mode of action in the treatment of hematopoietic disease.

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