Human hematopoietic microenvironments, *in vivo, in vitro* and on chip

by

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Abstract

My engagement in stem cell research started at the Norwegian Radium Hospital (1998-2004). Our group focused upon the regulatory role of the microenvironment toward hematopoietic cells in the bone marrow. By applying traditional life science methodologies (genetic engineering and methods for identification, isolation and cultivation of cells), we wanted to map and evaluate multiple factors controlling stem/progenitor cells. These elements have potential relevance for tissue engineering and cancer treatment.

Specifically, we were concerned with the nature of the supportive cells ("niche cells") that are indispensable for maintaining control of the hematopoietic cells. From an immunohistochemical study of bone marrow biopsies, we found that all stages of human B-cell progenitors co-localise with slender $CD10^{+}VCAM^{+/-}$ cells, indicating an essential role of this stromal phenotype (Article II).

In our search for proteins mediating communication between B-cell progenitors and their microenvironment, we employed a cloning strategy for identifying proteins displayed by pro B-cells (known as a signal sequence trap, SST). Three candidate proteins were detected and one unknown protein was characterised (TMEM-9). However, this protein was localised to internal cell membranes (also trapped by the method) with no obvious role in cell communication (Article I).

We also investigated Wnt signalling as a candidate pathway operating in the B-cell niche. This pathway has fundamental regulatory roles in hematopoiesis and other developmental processes. Human B-cell progenitors were found to transcribe essential molecules for conveying canonical Wnt signalling. Moreover, we observed that triggering of this pathway caused a characteristic intracellular event and a cease in cell differentiation and proliferation (Article III). This outcome was surprising, as the opposite result had been documented in the murine system. However, as Wnts are morphogens, they are likely to act in precise gradients to control cell behaviour *in vivo*. Hence, our current *in vitro* models may not provide physiological results.

Given access to microfabrication facilities at the Department of Micro and Nano Systems Technology (IMST) at the Vestfold University College (2006), I was able to pursue research on stem cell niches from a completely new angle. I explored the laminar

motion of fluids in microchannels and realised its potency for unique manipulation of cell cultures, with respect to both cellular composition and chemical environment. I figured that such strategies may be used for making more representative *in vitro* models of the hematopoietic niches in the bone marrow.

By implementing the soft lithographic process, I fabricated a platform for on-chip differentiation based on a published design. As a first step, I needed to test whether the microfluidic conditions were in accordance with normal cell behaviour. Differentiation of mesenchymal stem cells (MSCs) was chosen as a relevant test process, knowing that both undifferentiated MSCs as well as some of their specialised progeny are appropriate feeding layers for stroma-dependent hematopoietic cells. Substrate modifications were needed to obtain a device that accommodated MSCs in a healthy state during prolonged cultivation, as demonstrated by extensive adipogenic and osteogenic differentiation (Article IV). The successful management of MSCs cells on the microscale is an important preparation towards the aim for making artificial hematopoietic stem cell niches on a chip.

Acronyms

BM	Bone marrow
BMP	Bone Morphogenetic Protein
BCR	B-Cell Receptor
BSA	Bovine Serum Albumin
cDNA	complementary DeoxyriboNucleic Acid
CD	Cluster of Differentiation
CFSE	5- and 6- CarboxyFluorescein diacetate Succinimidyl Ester
cfu	colony forming units
CXCL-12	Chemokine Ligand 12
CXCR-4	Chemokine Receptor 4
Dkk-1	Dickkopf 1
ECM	ExtraCellular Matrix
FCS	Fetal Calf Serum
Flt3L	FMS-Like Tyrosine kinase 3 Ligand
Fzd	Frizzled
sFRP	secreted Frizzled Related Protein
G-CSF	Granulocyte-Colony-Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GSK3β	Glycogen Synthase Kinase 3 beta
HSC	Hematopoietic Stem Cell
Ig	Immunoglobulin
IL	Interleukin
L	Ligand
LEF-1	Lymphoid Enhancer-binding Factor 1
LIF	Leukemia Inhibitory Factor
LRP	Low-density lipoprotein Receptor related Protein
MNC	MonoNuclear Cells
MMP	Matrix MetalloProteinase
MSC	Mesenchymal Stem Cell
Pax5	Paired box gene 5
Pre B	Precursor B-cell
Pro B	Progenitor B-cell
r	recombinant
R	Receptor
RNA	RiboNucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCF	Stem Cell Factor
SDF-1	Stromal Derived Factor 1
TCF7L2	Transcription factor 7-like 2 (formerly TCF)
TdT	Terminal deoxynucleotidyl Transferase
TSLP	Thymic Stromal-derived LymphoPoietin
VCAM-1	Vascular Cell Adhesion Molecule 1
VLA4	Very Late Antigen 4
WIF-1	Wnt Inhibitory Factor 1
Wnt	Wingless

Preface

This thesis is submitted in partial fulfilment of the requirements for the degree of Philosophiae Doctor at the University of Oslo, Norway.

The work presented in this thesis was performed at the **Department of Immunology**, Institute for Cancer Research at the Norwegian Radium hospital in Oslo (Article I-III) and at the **Department of Micro and Nano Systems Technology** (IMST) at the Vestfold University College in Horten (Article IV). I gratefully appreciate the financial support from these departments.

I want to express my sincere gratitude to:

My insightful and encouraging supervisors **Edith Rian** and **Steinar Funderud**; Edith for her persistent enthusiasm, professionalism and ambitious research strategies, Steinar for introducing me to the fascinating field of stem cell research and for being a "scientific father".

Henrik Jacobsen and **Frank Karlsen**, who allowed me to enter the field of microtechnology and to build a cell lab at IMST.

My co-authors **Marit Kveine Nygren**, **Guri Døsen Dahl**, **Heidi Stubberud** and **Emina Torlakovic** for all their hard work and for being such a pleasant group of colleagues. **Lene Alsøe** for sharing her excellent expertise in cDNA library construction and for adding harmony to "Lab 4".

Anna Tourovskaia at the University of Washington for providing basis for the microcultivation studies. Her unconditional and reliable support, both technically and morally, is a praiseworthy example of generosity within the scientific community.

My **fellow PhD students**, **supportive staff** and **other colleagues** at both Radiumhospitalet and Vestfold University College for providing stimulatory working environments and for maintaining the lab facilities.

My brother, professor dr. med. **Olav Tenstad**, who told me to wrap up this work. I am grateful that my studies finally added up to a thesis, on behalf of the institutions that employed me, and of course personally. Performing research poses a challenge to a range of personal capabilities, of both intellectual and practical nature. There is a need for strategies to be decided, scientific contacts to be made and kept, materials and equipment to be provided, experimental procedures to be optimised and results to be scrutinized.

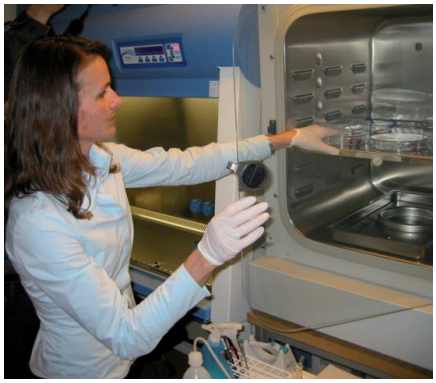
Moreover, without proper presentation to the world outside, textually and graphically, the work has limited scientific value. And most of all, there is a need for endurance. Put in poetic terms, developing skills in these areas may be like rewarding fruits, feeding a researcher while busy nurturing and growing our common tree of knowledge.

Although I have put all my professional energy into research, I have been conscious about not neglecting my family, first of all: Magne, Torstein, Amund and Erlend.

I dedicate this work to my kind father Ole Mathias Tenstad, who worked hard all his life for the benefit of his patients, and lost his fight against cancer in 2005.

Horten, April 2010

Ellen Tenstad



Errata

Corrections added in proof:

Location	*Submitted version*	*Corrected version*
Front page	Department of Medicine	Faculty of Medicine (Names and logos of departments are added)
piii	- evaluate the multiple - stem/progenitor cells..	- evaluate multiple - stem/progenitor cells.
piii, iv	- Institute for MicroSystem Technology (IMST) at Vestfold	- the Department of Micro and Nano Systems Technology (IMST) at the Vestfold
pv	Lymphoid Enhancer-binding Factor	Lymphoid Enhancer-binding Factor 1
pix	- Funderud S., - 13 - Rian E	- Funderud S, - 13. - Rian E.
p2	- myeloid and lymphoid - specialize	- lymphoid and myeloid - specialise
p3	Naural Killer	Natural Killer
p4	- compatible donor, (- a population of	- compatible donor (- the population of
p13	favours their	favours its
p17	Localization/Growht	Localisation/Growth
p24	died solution	dyed solution
p25	the use of	use of
p29	the first cycle serve to	the first cycle serves to
p30	induced and reversible	induced and irreversible
p33	TdT^+, cells respectively).	TdT^+ cells).
p42	numbers both in cultivation assays, as	numbers, both in cultivation assay as
p46	Tumor rejecting antigen	Tumor rejection antigen 1
p50	A recent review point out	A recent review points out
p51	A recent cultivation study implement	A recent cultivation study implements
p52	- B cell progenitor - BM tissue showed	- B-cell progenitors - BM tissue shows
p55	Two variant microfluidic system	Two variant microfluidic systems
p56	- demonstrated its - documentation of MSC differentiation	- demonstrated their - documentation of on-chip MSC differentiation
p57	the unique features of microfabricated tools	unique microscale features
p59	- localization - Visualization	- localisation - Visualisation
P68	Institute for MicroSystem Technology	Department of Micro and Nano Systems Technology

Additionally, some format changes have been implemented, e.g.:

Location	*Submitted version*	*Corrected version*
front page	*on chip*	on chip
pvi	hospital	Hospital
pv	Dickkopf-1	Dickkopf 1
p3++	- T cell - B cell	- T-cell - B-cell
p23	Wnt independent	Wnt-independent
p25	virus containing	virus-containing

List of articles

I. Kveine M, **Tenstad E**, Dosen G, Funderud S, Rian E.
"Characterization of the novel human transmembrane protein 9 (TMEM9) that localizes to lysosomes and late endosomes."
Biochem Biophys Res Commun. 2002; 297 (4): 912-917.

II. Torlakovic E*, **Tenstad E***, Funderud S, Rian E.
"$CD10^+$ stromal cells form B-lymphocyte maturation niches in the human bone marrow."
J Pathol. 2005; 205 (3): 311-317.

III. Dosen, G, **Tenstad E**, Nygren MK, Stubberud H, Funderud S, Rian E.
"Wnt expression and canonical Wnt signaling in human bone marrow B lymphopoiesis."
BMC Immunol. 2006 (7), 13.

IV. **Tenstad E**, Myklebost O, Rian E.
"Extensive adipogenic and osteogenic differentiation of patterned human mesenchymal stem cells in a microfluidic device."
Lab Chip. 2010, DOI: 10.1039/b926738g. (Due in May; issue 11).

* Both authors contributed equally to the manuscript.

Contents

1 Introduction

1.1 The stem cell concept

Stem cells are immature, unspecialised cells with two unique features:

1. They can mature into specialised cells with distinct "working tasks".
   This process is called cell differentiation.

2. They can make identical copies of themselves, thereby securing their continuation.
   This process is called self-renewal.

Stem cells constituting the early embryo are the basis for the development of an entire new individual. In completed organisms, stem cells are critical for maintaining the body's balance (homeostasis), as many specialised cells don't replicate and have short life spans. These stem cells are called somatic stem cells[1] and have less potency, as they normally only specialise into the cell types of the tissue from which they originated. They have been found in a variety of tissues, including bone marrow (BM), peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver and pancreas [1].

1.2 Developmental processes in the bone marrow

1.2.1 Hematopoietic stem cells

The red BM is an adult tissue with ongoing stem cell activity. This is the site[2] for the production of new blood cells (hematopoiesis), a process that is vital throughout life. In fact, blood cells are among those with the shortest survival period, e.g. neutrophil granulocytes only live for a few days and platelets live for ~10 days. Also, the adaptive immune system relies on a continuous production of immune cells with random specificity for being able to defeat unfamiliar pathogens. Remarkably, all blood cells originate from a single population of stem cells, called hematopoietic stem cells (HSCs),

[1] Also called postnatal or adult stem cells.
[2] In cases of stress, hematopoiesis also takes place in spleen (extramedullary hematopoiesis).

thereby qualifying as multipotent stem cells.[3] In fact, the ability of transplanted HSCs to rescue a mouse deprived of all its hematopoietic cells (by irradiation) is the gold standard for defining these cells (= reconstitution potential). Actually, a single HSC has been demonstrated to achieve this tremendous task [2]. Our ability to manage HSCs has great clinical impact; see **Box 1**, page 4.

HSCs were recognised more than 40 years ago[4] and are the most-studied type of adult stem cells. They are rare[5] [3] and cycle very slowly [4]. Yet, the production of mature cells is enormous - billions of new blood cells are produced each day. These numbers are explained by substantial cell amplification during the hematopoietic process. The HSCs give rise to a cascade of intermediate cell stages that gradually differentiate, meaning they acquire the specific properties of a particular mature cell type. These cells are known as progenitors and precursor cells, of which some have a vivid proliferation. Concomitantly, the partly differentiated cells loose potency, denoting that they become more restricted to evolve into a specific cell type (lineage commitment). The differentiation processes and lineage decisions are driven by activation and inactivation of specific genes in a strictly regulated fashion. Thus, HSCs and blood cells at different differentiation stages can be identified by the presence and absence of genetic elements and surface markers, as well functional criteria and morphology[6] (see section 1.4.1, page 13).

Studies on hematopoietic cells of different maturation levels give rise to "roadmaps" that describe anticipated hierarchical lineage commitments. A traditional overview is shown in **Figure 1**, assuming that the first differentiation step produce lymphoid and myeloid precursors [5, 6]. The lymphoid precursors give rise to B-cells, T-cells and natural killer (NK) cells, whereas the myeloid precursors give rise to red blood cells, platelets, granulocytes, macrophages and osteoclasts. Dendritic cells (DC) seem to originate from both precursors. However, this classical road map for lineage commitment is regularly challenged by findings of progenitors with mixed potential [7-9]. Additional heterogeneity to the picture emerge from clonal analysis revealing that individual stem cells exhibit lymphoid/myeloid biases that are heritable through multiple rounds of

[3] Multipotency: the ability of a cell to specialise (differentiate) into a limited number of cell types or into closely related family of cells.
[4] HSCs were postulated by Artur Pappenheim (1917), experimentally confirmed by Alexander Maximow (1924) and demonstrated by Till and McCulloch (1961).
[5] HSCs constitute approximately 1: 10 000 BM cells.

transplantation [10]. Finally, it is suggested that stemness is a biological function that degenerates over time but may be recruited within differentiated cells in particular contexts [11, 12].

Blood cells leave and enter the BM via an elaborate network of small, highly permeable blood vessels called sinusoids. T-cell precursors and immature B-cells continue their development in the thymus and spleen, respectively.

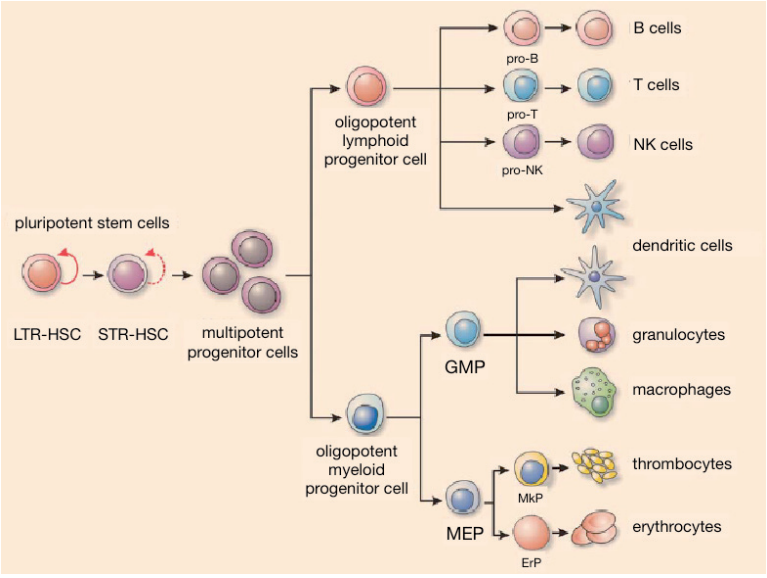


Figure 1 Simplified model of hematopoiesis HSCs can be divided in Long-Term Repopulating (LTR)-HSCs and Short-Term Repopulation (STR)-HSCs, which can self-renew (indicated by red arrow). They give rise to multipotent progenitor cells, which differentiate to all blood cells via several progenitor cell stages of less potency. A "developmental tree" is perceived when the figure is rotated 90˚. The stem cell populations represent the stem of the tree, while progenitors and mature cells constitute branches and leaves. ErP, erythrocyte progenitor cell; GMP, Granulocyte-Macrophage Progenitor cell; MEP, Megakaryocyte-Erythrocyte Progenitor cell; MkP, Megakaryocyte Progenitor cell; NK, Natural Killer. Modified from [13].

Obviously, the potent process of hematopoiesis must be tightly and dynamically regulated according to the changing needs of the organism. Self-renewal and differentiation of HSCs need precise tuning in order to maintain the stem cell pool while all the specialised blood cells are generated in right proportions. This demand leads focus towards the HSC microenvironment.

[6] Morphological identification is only reliable for some mature cells.

Box 1 Realizing the potential of HSCs – a lesson from the clinic

The central features of HSC are demonstrated by the symptoms and treatment of patients suffering from aplastic anaemia. In these patients, the HSCs fail to produce blood cells, resulting in anaemia, bruises and enhanced susceptibility to infections, due to low numbers of red blood cells, platelets and immune cells, respectively. The patients can be saved by a transplant of healthy HSCs from a compatible donor (notably after their own distorted hematopoietic system has been eradicated by irradiation or chemotherapy). The HSCs are injected into a peripheral vein and migrate to the BM (= homing) where they settle (= engraftment) and repopulate the hematopoietic system. This strategy is also used to fight blood cancers. [14, 15]

1.2.2 Mesenchymal stem cells – relation to hematopoiesis

In addition to hematopoietic cells, the BM tissue consists of a variety of cell types including adipocytes (fat cells), osteoblasts (bone forming cells), endothelial cells, fibroblasts and reticular cells [16]. Because the tissue is soft, containing little extracellular matrix, human BM cells are harvested by aspiration, commonly from the iliac crest. When the mononuclear cell (MNC) fraction of a BM aspirate is seeded the *in vitro*, the population of non-hematopoietic cells is revealed, being plastic-adherent. Studies on these cells started shortly after the discovery of HSCs. It was found that they contain a small fraction of undifferentiated cells having a fibroblast-like morphology and stem cell properties; 1) they were clonogenic[7] and 2) they were able to differentiate; when individual clones were transplanted into a host animal (i.e. *in vivo*), they formed bony fragments [17, 18]. Importantly, the regenerated bone structures (originating from donor cells) were shown to encase hematopoietic cells of the host [18-20]. Also, when a sample of whole marrow was seeded in a culture dish (i.e. *in vitro*), a close association between hematopoietic and non-hematopoietic BM cells was observed. It was found that the non-hematopoietic BM cells were necessary for growing cultures of HSCs and make them differentiate into to myeloid [21] and lymphoid [22, 23] cells. These and supportive studies have led to two important concepts [24]:

[7] Clonogenic: ability to generate genetically identical cells, an ability shared by stem cells and committed progenitors

1. The BM contains non-hematopoietic stem cells that are multipotent, giving rise to various lineages, including osteoblasts, adipocytes and chondrocytes [25, 26]. These cells are most commonly known as mesenchymal[8] stem cells (MSCs, **Box 2**).

2. The tissue formed by MSCs provides an adequate microenvironment for HSC homing and growth [27].

Thus, MSCs have a central role in both maintaining the structure of the BM tissue and support hematopoiesis. The heterogeneous population of MSCs and their progeny is called stromal cells[9] (stroma is the Greek word for mattress) and the regulative roles of the microenvironment are presented below (see sections 1.3 and 1.4.2). The BM is the only known organ in which two separate and distinct stem cells and dependent tissue systems not only coexist, but functionally cooperate [20], see **Figure 2**. It has been speculated whether there is a common precursor in adult for mesenchymal and hematopoietic lineage cells, but this remains controversial [28, 29].

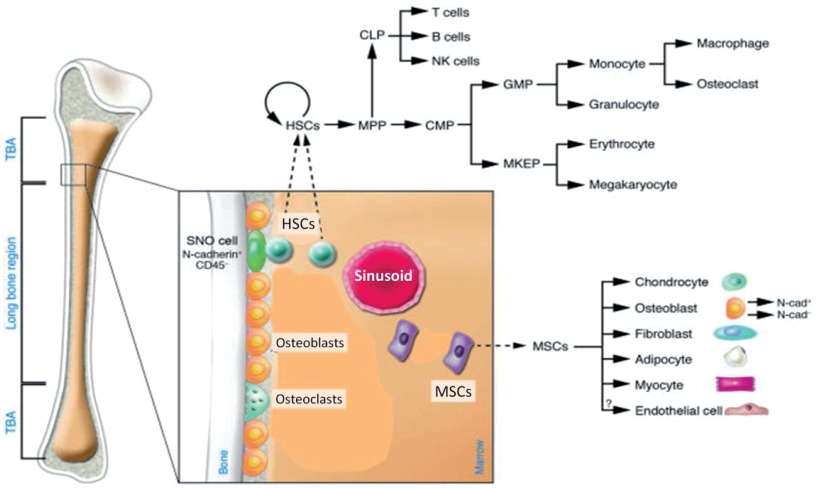


Figure 2 Developmental processes in bone marrow Modified from [30]

[8] Mesenchymal: originating from the mesoderm in the embryo, refers to cells that develop into hemaopoietic and connective tissue. However MSCs do not differentiate into hematopoietic cells.
[9] Stromal cells is a collective term for all the different supporting cells found in a given tissue, as distinguished from the functional elements of this tissue or organ (parenchymal cells). Confusingly, this term frequently refer to the MSC population alone (Box 2)

Box 2 MSC biology

Nomenclature Dozens of names have been given to primitive populations of adherent BM cells, including:

- *colony-forming unit fibroblast (CFU-F)[19]*
- *mesenchymal progenitor cells [31]*
- *BM stromal stem cells[20]*
- *BM derived multipotent mesenchymal stromal cells[29]*
- *skeletal stem cells [32]*
- *stromal precursor cells[33]*
- *BM stromal cells [34]*
- *BM osteogenic stem cells [35]*

A consensus statement suggests that the acronym MSC can be used in general, however, whether it refers to "mesenchymal stem cells" or "multipotent mesenchymal stromal cells" must be defined by each investigator [36, 37]. The self-renewing capacity of a MSC population ($CD146^+$ CFU-Fs) has been demonstrated experimentally just recently [27], thereby crediting use of the "stem cell"-term for these cells.

Identification criteria No marker is MSC specific, thus 3 criteria has been proposed to define MSCs [36]:

1) Plastic adherence

2) Phenotype: Positive for CD105, CD73, CD90; Negative for CD45,CD34, CD14, HLA-DR or CD11b and CD79a or CD19

3) *In vitro* differentiation to osteoblasts, adipocytes and chondrocytes, **Figure 3**

More recently, CD146 is suggested as a MSC marker [27, 38]. However, the MSC phenotype is elusive, as it seems to be constantly changing in response to their microenvironment, both *in vivo* and *in vitro* [20, 39].

Developmental plasticity MSCs has been reported to differentiate into non-mesenchymal cells, e.g. pancreatic, liver and lung cells (endodermal) and neurons and skin cells (ectodermal). However these results may represent experimental artefacts [40] and are controversial [41, 42], **Figure 3**

MSC numbers and distribution BM MSCs are rare, ranging from $1/10^4$ to $1/10^5$ BM MNC [43]. Cells with MSC characteristics are found in a variety of post-natal organs and tissues including: brain, spleen, liver, kidney, lung, BM, muscle, thymus, pancreas, blood vessels, fat, skin, as well as in fetal tissues [44-46] and circulate in peripheral blood in low number [29]. However, MSC from different sources are not considered equivalent.

Clinical interest Reports of transdifferention have suggested that MSCs could be used for the regeneration of almost any tissue. MSCs transplantation has been explored for a number of conditions, but the differentiation potential of MSCs has only been successfully exploited to treat bone disease [47, 48]. However, MSCs have a crucial immunomodulatory role, which contributes to reducing inflammation and inducing regeneration, thus showing potential for treating conditions like graft-versus-host disease, GVHD [47]. Recently, BM stromal cells (i.e. MSC progeny) have been implicated in regulation of immunological memory, by organizing defined numbers of dedicated survival niches for plasma and memory T-cells in the BM [49].

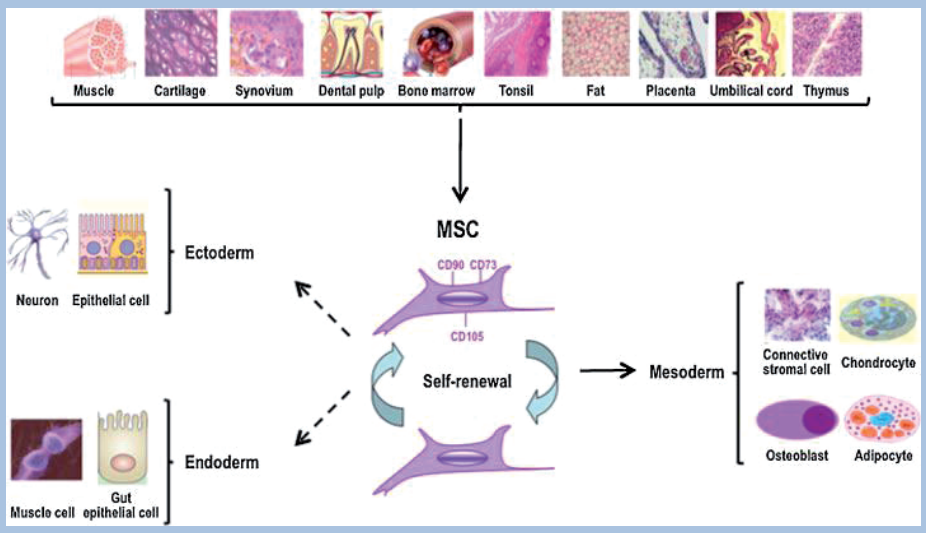


Figure 3 MSCs; sources, self renewal and differentiation potential
Dotted lines indicate uncertainty. From [41].

1.3 The hematopoietic microenvironment. Stem cell niches

In recent years, the discovery of "stem cell niches" has led special focus towards the cellular microenvironment, **Box 3**. However, the niche concept was established already in 1978 [50]. Experiments indicated that HSCs suffered from a loss in "immortality" when transplanted outside the BM (e.g. the spleen). Thus, an explanation was sought to understand why some stem cells are allowed to self-renew, while others propagate to the differentiation programs. It was postulated that stem cells reside in specialised anatomic sites of limited size (niches) where they are sheltered from differentiation signals, thereby ensuring their self-renewal. Nevertheless, the existence of stem cell niches was not

revealed until the turn of the millennium, when they were demonstrated in the invertebrate model Drosophila [51, 52]. Later, stem cell niches have been located in the BM, the epidermis, the intestinal epithelium, the neural system and the gonads (reviewed in [53]).

Box 3 The cellular microenvironment

The realisation of a cell's potential (proteome) depends as much on the cell environment as on the genetic material of the cell itself [54]. In the body, the different cells are surrounded and influenced by distinct systemic and local components that are carefully maintained. Included in each cell's specific microenvironment may be parts of the blood, lymphatic and nerve systems, other cells, ECM and interstitial fluid. The microenvironment varies in time and space and is essential for regulating cell behaviour; apoptosis, proliferation, differentiation and migration. The regulation is executed by mechanical and chemical stimuli. The chemical stimuli are transferred via diverse signalling substances, ranging from soluble molecules (endocrine, paracrine and autocrine signals) to insoluble molecules (ECM-components and membrane-bound proteins on neighbouring cells).

Microenvironment =
- Signalling molecules (e.g. cytokines)
- Extracellular matrix (ECM)
- Cell-cell contact
- Mechanical stimuli
- Physical parameters (gas, nutrients etc.)

1.3.1 HSC niches

Endosteal HSC niche Many studies drew attention to the bone forming cells, osteoblasts as a potential niche cell for HSC. Since 1975, many rodent studies had shown that HSCs locate close to the inner surface of the bone cavity (endosteum) - which is lined with osteoblasts [55-57]. Moreover, human osteoblasts could support HSCs *in vitro* (by production of cytokines; including Granulocyte Colony stimulating Factor (G-CSF), Granulocyte/Monocyte Colony Stimulating Factor (GM-CSF) and Leukemia Inibitory Factor, LIF [58, 59]) and *in vivo*; co-injection of osteoblasts improved HSC engraftment during transplantation [60]. Also, mouse mutants made unable to develop osteoblasts (and hence bone) had no BM, indicating that osteoblasts are required to initiate BM hematopoiesis [61].

In 2003, two mouse models served to demonstrate that osteoblasts do provide niches for BM HSCs and regulate their numbers dynamically. These mice had genetic modifications[10] making it possible to expand their osteoblast numbers. This was done either by over-expressing the receptor for parathyroid hormone (PTH[11]) [62], or by inactivating the receptor for bone morphologic protein 1 (BMPR1A) [63]. In both models, HSC numbers increased in parallel with osteoblast numbers. Another adult mouse model showed the opposite; when developing osteoblasts were induced to die, there was a loss of hematopoietic cells in the BM [64]. Interestingly, ablation of more mature osteoblasts had no effect on hematopoiesis, indicating that niches comprise immature osteoblasts [65]. Immunohistochemistry revealed that the HSCs were located together with **O**steoblasts that had **N**-cadherin on their surface and a **S**pindle shaped morphology; SNO-cells [63, 66] see **Figure 2**, page 5.

Vascular HSC niche Other indications pointed towards endothelial cells as candidate HSC niche cells. As HSCs are able to mobilise into the blood stream[12] within minutes [67], they should be closely associated with the sinusoids. Also, endothelial cells were found to support HSCs in culture [68, 69]. Endothelial HSC niches were acknowledged in 2005, when the majority of immunostained BM HSCs was shown to be in contact with the endothelium of the sinusoids. The BM tissue sections were taken from a mouse in steady-state, undermining the argument that the HSC-endothelial interaction was transient. These stainings were facilitated by the discovery that murine HSCs could be identified by a limited set of markers, due to their specific expression[13] of Signalling Lymphocyte Activation Molecule (SLAM) family receptors, i.e. they are $CD150^{+}CD48^{-}CD41^{-}$ [70].

Relation between HSC niches HSC states. Phenotyping and assays measuring cell division (label retaining assays[14]) have implied that HSCs harbouring the endothelial niche are more activated than those in the endosteal niche [71]. This finding favours the hypothesis that HSCs situated near the sinusoids serve to respond rapidly to the need for new blood cells (conveyed by blood-born factors), while the endosteal niche represent a

[10] Some genetic modifications are incompatible with normal development. However if they are made inducible, their effects can be studied in adult animals (conditional mutants).
[11] PTH is a clinically approved drug for the treatment of osteoporosis in humans
[12] HSC mobilisation is induced by G-SCF. Enables collection of transplantable BM HSCs from blood.
[13] The differentiated expression of SLAM receptors in HSC and MPP was found by comparing gene expression profiles.

"storage" niche. A quiescent nature of HSCs located in the endosteal niche has been supported by the finding that HSCs can be enriched from bones. HSCs isolated by enzymatic treatment of grinded bones were shown to possess a higher reconstitution potential than HSCs flushed from the central marrow [58, 72]. High reconstitution potential is a property linked to the most immature, nondividing HSCs, in contrast to those being more active and primed for differentiation. These populations are known as long-term populating HSCs (LTR-HSC) and short-term repopulating HSCs (STR-HSCs), see **Figure 1**, page 3. Initially, HSC quiescence in endosteal niches was believed to be mediated by N-cadherin on both HSC and osteoblasts, linking them together by homophilic adhesions [66, 73]. The presence of N-cadherin on HSC has been questioned [74], but a recent study detects intermediate and low levels on LT-HSCs and suggests that "reserved" HSCs express intermediate levels, while cells primed towards STR-HSCs express low levels [75].

Unifying links between the endothelial and the endosteal niche have been revealed. First, immunostainings showed that HSCs in both locations are in contact with reticular cells, a cell type that was top ranked in terms of CXCL12[15] secretion. (Such co-localisation applies to the most immature B-cell progenitors as well, see last paragraph, page 50). This chemokine binds to its receptor, CXCR4[16] on HSC, providing homing and essential support. The reticular cells were named CAR cells, for **C**XCL12 **A**bundant **R**eticular cells and supposed to be of osteogenic lineage [76, 77]. Second, there is a high chance for the two niches to be in close proximity, as red BM is highly vascularised. Indeed, it has been suggested that osteoblasts and endothelial cells *collectively* form the HSC niche [78]. Recently, this proposal was supported by a microanatomical investigation of the BM inside the murine scull. 3D visualisation[17] showed that over 90% of the BM is within 20 um of a blood vessel. This finding suggests that HSCs being on the endosteal surface are simultaneously influenced by vascular/perivascular cells, i.e. *that the endosteal nice is perivascular.* Impressively, transplanted HSCs were tracked over time in living mice, enabling the first observations of mammalian stem cell proliferation [79].

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[14] Slowly proliferating cells are visualised in situ by pulse labelling of their DNA (e.g.BrdU) and chase after month or more. This detection requires cell fixation, precluding subsequent functional analysis.
[15] CXC-chemokine (CXC) ligand 12. Also known as stromal cell-derived factor 1 (SDF-1), or pre-B-cell-growth stimulating factor (PBSF)
[16] CXC-chemokine (CXC) receptor 4. Entry receptor for HIV-1.
[17] 3D visualisation was enabled by multiple images acquired at 1 um steps

Other cells influencing the HSC niche(s) Adipocytes are very abundant in BM. They appear when hematopoiesis is initiated, both *in vitro* (own observations) and *in vivo* (during reconstitution regime) [80], indicating a significant role during this process. Adipocytes produce several cytokines that are critical to hematopoiesis (IL-6, IL-8, prostaglandin, leptin) [80] and are one of the stromal cell populations that produce adiponectin, a recently defined HSC growth factor [81]. Murine mutants lacking the adiponectin receptor have no HSC proliferation, implying an essential role of adiponectin. Other BM cells reported to regulate the HSC niche includes chondrocytes [82], nerves [78] and hematopoietic cells, such as monocytes and their derived osteoclasts [83]. Osteoclasts are specialised bone resorbing cells that derive from the myeloid lineage of hematopoietic cells.

Regulatory factors in HSC niches A range of molecules are responsible for organising the HSC niches, i.e. attract HSCs to the niche cells (chemokines), make them dock there (adhesion molecules) and receive the fate-determining signals. In concert, these factors allow stem cells to amplify or differentiate in adequate numbers, **Figure 4**. The following factors have been implicated in the functional dialog between HSC and their niche (incomplete list) [78, 80, 84, 85]: CXCL-12/CXCR4, N-Cadherin [75], osteopontin [86], multiple signalling pathways; Notch, Hedgehog [87, 88], Tie2/Angiopoietin-1 [89] and Wnt (section 1.5.2), as well as a number of cytokines, such as interleukin (IL)-3, IL-6, IL-11, FMS-like tyrosine kinase 3 ligand (Flt3L), thrombopoietin (TPO, [90]), G-CSF, GM-CSF, stem cell factor (SCF) and transforming growth factor-β1 (TGF-β_1). Additionally, factors regulating the niche cells may affect HSC indirectly (e.g. effect of BMP and PTH on osteoblast numbers). Oxygen tension is a physical parameter that has been related to HSC function. It is suggested that HSCs reside at the lowest end of an oxygen gradient and that hypoxia regulates vital cell functions and limits oxidative stress [80, 91].

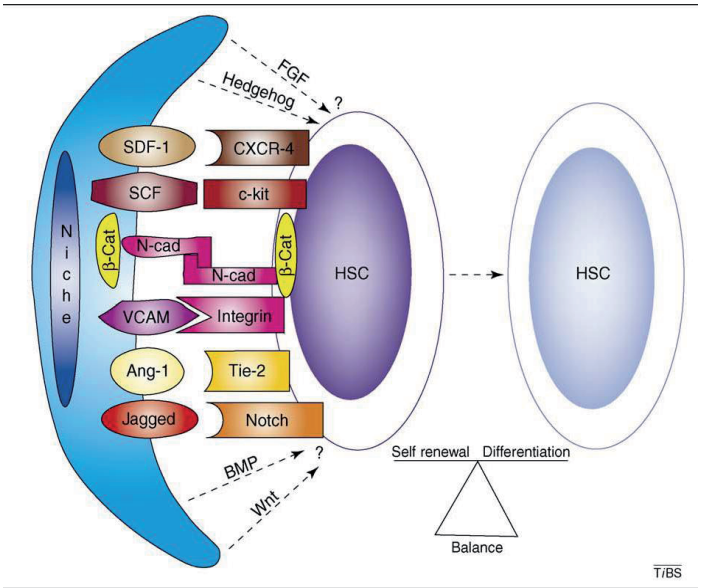


Figure 4 Niche signalling To maintain physiological homeostasis the niche orchestrates a myriad of signals to achieve a delicate balance between HSC self-renewal and differentiation. Depicted here are some of the various extrinsic regulatory factors originating from the niche, or more generally the BM microenvironment. Some ligands may have distinct or multiple or sources. From [92].

1.3.2 MSC niches

Several studies suggest that MSCs reside in a perivascular niche in almost all adult tissues. In fact, it has been suggested that MSCs may be identical to the pericytes (also known as adventitial cell, Rouget cells or mural cells)[18], according to data on common localisation, markers, differentiation potential and multipotency maintenance [39, 41]. Results in line with this view suggest that CD146 is a useful MSC-marker [27, 38, 93]. A perivascular localisation facilitates migration of MSCs to local or distant tissues in response to injury or pathogenesis [41].

[18] Mural cells wrap around blood vessels and are contractile cells which regulate vessel diameter and consequently blood flow. On large vessels they are multi layered and referred to as smooth muscle cells. Mural cells on smaller vessels are more sparse and is usually referred to as pericytes

1.4 Early B-lymphopoiesis

Formation of immature lymphocytes in the BM is called early lymphopoiesis. Further maturation takes place in the periphery and requires the presence of specific antigen.

The below presentation has a slightly practical perspective, as central characteristics of human B-cell development are provided by focusing on how B-cell progenitors are identified and cultivated. This knowledge is essential for pursuing investigation on early B-cell lymphopoiesis. Without knowing the cellular phenotype (what the cells look like), or which factors they depend upon, they cannot be identified, nor isolated or kept alive outside the body *(ex vivo*/*in vitro*) for functional studies. The spotlight is on human B-cell progenitors (according to Article I-III), including some comparisons to the murine system.

1.4.1 How to identify B-cell progenitors

B-lymphocytes protect us against pathogens by producing antibodies (Abs). These molecules are also known as immunoglobulins (Igs) and serve by binding to specific sites (antigen epitopes) on the surface of a pathogen. This "tagging of the enemy" favours its destruction by other immune cells (T-cells, macrophages). In our genome, we have several gene segments for the different chains of an Ig molecule; V, D and J segments in the H chain locus and V and J gene segments in the L chain loci. During development, each B-cell combines and link these genes (combinatorial rearrangement) [94]. If successfully processed, the cell ends up[19] producing functional Ig molecules with the ability to bind a unique epitope. This "gene-shuffling" takes place a-priori to pathogen confrontation and equips us with a population of mature B-cells (plasma cells) which harbour an Ab-repertoire that can fight all potential enemies.

The Ig-gene loci are rearranged during the early stages of B-cell development, while the cells are staying in the BM. Hence, the configuration of Ig genes serves to define several sub-populations of B-cell progenitors, each at different developmental stages [95]. These stages are further characterised by the presence or absence of multiple surface and nuclear markers. Altogether, the developmental stages of B-cell precursors can be identified by the molecules categorised in **Table 1** below. **Box 4** shows the

[19] Only B cells that achieve successful gene rearrangements survive.

markers used in this study for phenotyping HSCs and B-cell stages and includes a technical consideration.

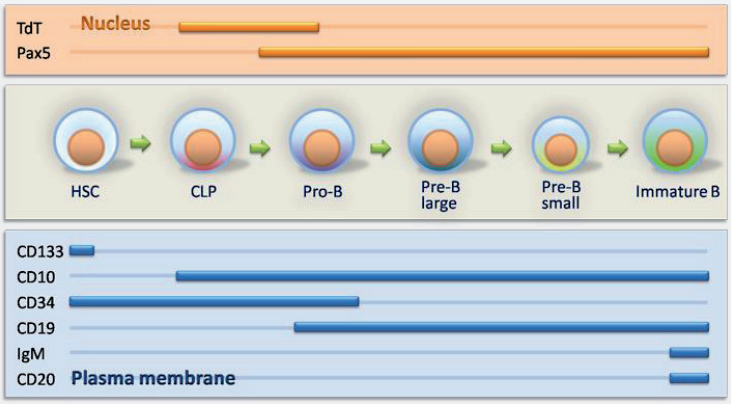
Recently, B-cells at different developmental stages have been subjected to large scale mRNA profiling [96]. Investigation of the presence/absence of mRNA species in distinct B-cell subpopulations enables the identification of new candidates for stage-specific markers as well as factors with potential impact upon the developmental process.

Table 1 Characteristic molecules for identifying and staging B-cells

Nuclear molecules
1 **Ig-gene** configuration.
2 Transcription factors, such as the **Pax-5** protein, being one of at least 10 factors governing the early stages of B-cell development [97], see **Box** 5
3 Enzymes specific to developing lymphocytes; such as: • **TdT**; Terminal Deoxynucleotidyl Transferase, an enzyme contributing to Ig-gene diversity by adding nucleotides, and • **RAG-1** and **RAG-2** protein, originating from Recombination Activating Genes, enzymes that are essential for the gene rearrangements.
Surface molecules
4 Ab molecules **pre BCR** and **BCR**[20] ("preliminary" and "final" B-cell receptor) The ligand binding part of BCR is a membrane-bound IgM or IgD molecule.
5 CD molecules, such as **CD19** and **CD20** [98].

[20] The pre-BCR consists of μ H chains and a surrogate L chains (composed of λ5 and VpreB proteins) whereas the final BCR consists of μ H chains and L chains.

Box 4 Identifying markers on HSCs and B-cell progenitors



Technical consideration When sorting living cells for the purpose of functional assays, the method used for cell isolation must maintain the cellular membranes intact. This premise precludes identification of specific cell populations by immunolabelling of intracellular markers. Because of their molecular size, the Abs used for detection cannot pass the plasma membrane unless this barrier has been ruptured by a fixation regime. Thus, living B-cell progenitors are identified by their specific expression pattern of surface markers. In contrast, fixed cells or cryo-sectioned tissue can be searched for all markers as these preparations expose all molecules; those bound to the plasma membrane as well as those situated in the cytoplasma or nucleus.

Box 5 B-cell transcription factors

The transcription factors act in concert to control B-cell differentiation, as demonstrated by murine knock-out experiments. **Ikaros** and **PU-1** promote commitment into the lymphoid lineage, while **E2A** and **EBF** co-act to initiate expression of many B-cell specific genes, including the master gene **Pax5**. Its product, the **Pax 5-protein** (formerly **BSAP**) emerges at the pre-pro B-stage and is present in all B-cell stages except at the final plasma cell stage. By dictating the expression of about 170 genes, Pax5-protein controls B-cell development and function (e.g. B-cell signalling, adhesion and migrating of mature B-cells). Also, the Pax 5-protein is considered to repress genes giving rise to other blood cells; when Pax-5 is deleted from pro B-cells, they can develop into other blood cells (at least *in vitro*). Thus, Pax5 has been called the "guardian of B-cell identity and function" [97-101].

1.4.2 How to cultivate B-cell progenitors

B-cell progenitors from adult human BM, as well as the most immature progenitor cells, LTC-IC[21] are not easily kept in culture. This finding substantiates our understanding of their intricate dependency on a balanced combination of environmental factors in the body. No one has been able to identify a cytokine-cocktail that alone supports these cells properly in culture [102, 103]. However, assisted by growth factors (see below), they can be successfully grown on a feeder layer of stromal cells that provides uncharacterised stimuli, see right panel of **Figure 15**, page 45. Thus, it is likely that critical stimuli are mediated by both soluble factors *and* adhesive interactions with the microenvironment (stromal cells and their ECM products). The importance of adhesive interactions is supported by poor progenitor survival when physically separated from the feeder layer by the use of a membrane [104]. Stromal cells may also function as a "docking station" facilitating homotypic interactions between hematopoietic cells [105].

Empirically, the potency of hematopoietic stem/progenitor cells is inversely related to age, and cells originating from unborn individuals have been maintained in less complex systems. Using progenitors harvested from human fetal liver, B-lymphopoiesis has been achieved in serum-deprived, stroma-free cultures [106]. Conversely, a fetal system, has allowed human HSCs to differentiate into immature B-cells using stromal cells solely (no serum, no cytokines added). However, no proliferative expansion was obtained, suggesting some missing factors [107].

Stromal phenotypes The first cultivations showed that *murine* B-lineage cells (as well as HSCs) could be supported by adherent cells from the same BM sample [21, 22]. This strategy applies to *human* B-lineage cells as well. Effective stromal supporters include human endothelial cells (HUVEC) and murine cell lines of different origins; e.g. AFT024 [108], MS-5 [109], S17 [110] and OP-9 [111]. Recently, primary MSCs and two MSC cell lines (hTERT-MSC and iMSC#3)[22] have qualified as B-lineage supporters [112, 113] and own observations. Also, osteoblasts have been shown to support B-lymphopoiesis in vitro [114].

[21] LTC-IC: **L**ong-**T**erm **C**ulture **I**nitiating **C**ells. The LTC-IC assay quantifies primitive hamatopoietic cells (Coulombel, *Oncogene,* 2004. 23, p 7210).
[22] both immortalized with telomerase reverse transcriptase (hTERT). They display an MSC-like morphology and have maintaidned ability to differentiate to osteoablasts, adipocytes and chondrocytes.

Not surprisingly, various stromal populations support B-lineage cells and other hematopoietic cells with different efficacies and different clones may even show different support towards different stages of B-cells [115]. Investigations of different stromal populations have not resulted in a clear consensus on the phenotype of the "B-cell-supporting stromal cell(s)". A subtractive gene expression strategy has revealed that stem cell-supporting stromal cell lines selectively express hundreds of gene products. Evaluation of these products suggests that the stromal cells are immature, sessile and highly reactive after binding to integrin ligands and cytokines [116]. However, similar to what applies to HSC niches, it is likely that B-cell progenitors in their native BM microenvironment may receive signals from various cell types (see section 4.4, page 48).

Factors regulating B-cell progenitors When cultivating adult human B-cell progenitors, cytokines are normally supplied in addition to the stromal feeding layer. These factors act alone or in synergy to enhance cell survival, proliferation and differentiation. Factors reported to influence the generation of human B-lineage cells are listed in **Table 2**. Additionally, Notch signalling determines the fate decision of lymphocyte precursors; low doses favour B-lineage differentiation whereas high doses favour T lineage differentiation [111, 117]. For the effect of Wnt signalling, see page 22.

Table 2 Factors influencing the generation of human B-cell progenitors

B-cell factor	Stromal factor	Effect
c-kit (CD117)	kit-ligand/ SCF	↑[109]
Flt3 (CD135)	Flt3 L	↑[118, 119]
G-CSF R	G-CSF	↑[109]
IL-7 R (CD127)	IL-7	0 [107, 120] ↑[119, 121]
IL-3 R (CD127)	IL-3	↑[122] ↓[123]
CXCR4 (CD184)	CXCL12 (SDF-1)	Localisation/Growth [124]
VLA4 (CD49D)	VCAM-1 (CD106)	Localisation/ Growth [125-127]
TSLP-R	TSLP	↑[125] 0 [98, 119]
BMP-R	BMP-6	↓[128]

↑: promoting effect, ↓ negative effect. See references for precise action (i.e. on proliferation, differentiation or survival). Full names are given in Acronyms.

Although murine B-lineage cells are commonly co-cultivated with stromal layers, their demands are better characterised and murine B-lymphopoiesis can even be performed without stromal cells and serum, given the presence of three cytokines; IL-7, Flt3L and SCF [102]. Such a protocol enables direct evaluation of culture manipulations (e.g. addition of other factors).

1.5 Wnt signalling

According to their phenotype, cells are equipped with specific receptors on their surface, making them receptive to certain signals present in their microenvironment (**Figure 4**, page 12). Typically, the binding of a given signalling molecule to its designated receptor results in transcription of dedicated genes, thereby directing cell behaviour. However, the path towards this end is commonly controlled by a range of molecules, forming a cellular pathway. Moreover, different pathways usually interact in signalling networks. Such complexity is certainly demonstrated in Wnt signalling, which has been studied intensively over the last years.[23]

Wnt signalling has fundamental instructive roles in both development and maintenance of an organism by governing vitals such as polarity and cell fates (proliferation, differentiation and apoptosis). Thus, malfunction of this signalling can lead to cancer and degenerative diseases [129, 130]. The molecules involved in the pathway (**Figure 6**, **Table 3**) are highly conserved between species, demonstrating their significance in evolution [131]. Triggering of the pathway is initiated by Wnt molecules, which are characterised as morphogens, see **Box 6**.

The human genome has a broad repertoire of both Wnt molecules (n=19) and their Frizzled receptors (n=10) and their specificities are partially promiscuous [136]. Wnts are glycoproteins harbouring lipid modifications being essential to their function [137]. Hence they have limited solubility and tend to act locally. They are able to activate several signalling cascades in the cell, known as the “canonical” pathway and different “non-canonical” pathways. Hence, they have been categorised according to which pathway they were thought to activate. But, as one single Wnt protein seem to have the capacity to activate multiple pathways, the receptors are more likely to determine the outcome of the Wnt activity [138]. The canonical Wnt pathway controls cell differentiation, proliferation,

and apoptosis by regulating expression of a high number of target genes [139]. The non-canonical pathways have traditionally been considered to control cell movement and tissue polarity [140]. However, the picture is highly complex at many levels as the Wnt proteins may interact with other factors in the extracellular space [141] and have receptors other than Frizzled [136]. Furthermore, the Wnt pathways cross talk both with each other [142] as well as other pathways [144, 145] and the intracellular pathway molecules can have multiple functions [146]. For instance, β-catenin is both involved in cell adhesion and nuclear events, see below and **Figure 17**, page 52.

Box 6 Morphogens

A morphogen is a signalling molecule that elicits different cellular responses depending on its concentration, thus governing tissue morphology. Morphogens spread from localised sites of production by diffusion. Distinct morphogen concentrations stimulate target cells to transcribe different combinations of the responsive genes, which in turn specify cellular fate. A cell's perception of morphogen concentration is thought to be determined by the number of active receptors [132, 133]. The concept of morphogens was originally defined by the mathematician A.M Turing in 1952 [134].

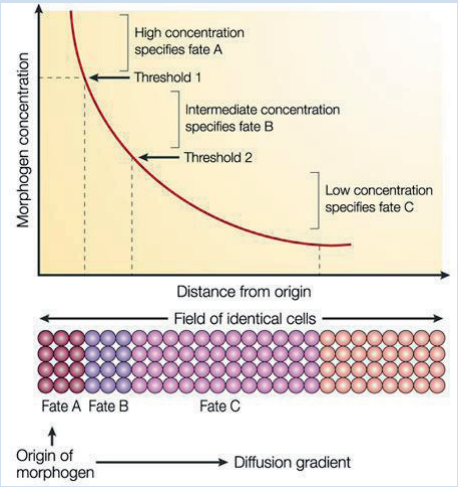


Figure 5 Morphogen concept
From [135]

1.5.1 The canonical Wnt pathway

The canonical Wnt pathway (also known as the Wnt/β-catenin pathway) is the one best understood. It can be described in short as follows. At the cell surface, a Wnt molecule is recognised by a receptor complex, consisting of Frizzled and LPR. The receptor-ligand interaction allows for cytosolic accumulation of the key player β-catenin in a process

[23] Discovered nearly 20 years ago, Wnts are mentioned in nearly 5000 journal articles listed on PubMed, over half of which were published in the past 3 years (Gordon, Journal of Biological Chemistry, 2006, 281, p22429).

thought to involve Dvl. β-catenin molecules pass through the nuclear membrane and stimulate transcription of the Wnt target genes [139] by binding to the transcription factors LEF-1 or TCF and replacing the repressor Groucho.

In absence of a Wnt signal, unbound β-catenin molecules in the cytosol are constantly targeted for degradation. Facilitated by scaffolding proteins (APC and Axin), β-catenin is first phosphorylated (by the kinases GSK3β and CK1), and then ubiquitinated (by a ligase complex, containing β-TRCP). Consequently, β-catenin is degraded by the proteasome and cannot stimulate transcription.

Additional control of the Wnt pathway is mediated by soluble inhibitors. Dkk-1 is able to block the Wnt receptor signal by interfering with the LPR co-receptor. WIF-1 and sFRPs (secreted forms of Fzd) can bind and confiscate Wnt proteins.

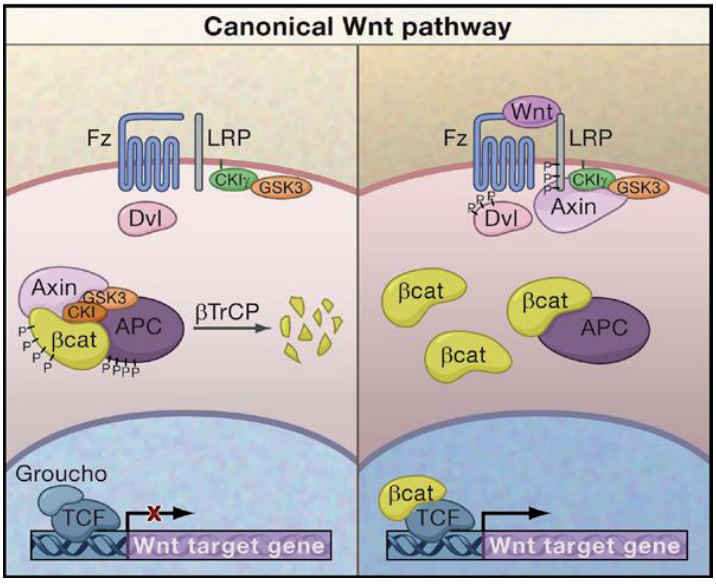


Figure 6 The canonical Wnt pathway. Right panel: A Wnt signal at the cellular surface is conveyed by β-catenin into transcription of Wnt target genes. The ligand-bound receptor inactivates the factors responsible for β-catenin degradation. Left panel: Without Wnt present, β-catenin is degraded and cannot stimulate transcription. Drawing from [130].

Table 3 Molecules involved in the canonical Wnt pathway

Molecule	Full name	Action
Roles in activation of Wnt target genes		
Wnt ligands	(From Wingless and INT-1)	Bind Fzd receptors and activate Dvl
Fzd (or Fz),	Frizzled	Bind Wnt ligands
LRP, (variants)	Low density Lipoprotein Receptor-related Protein	and activate Dvl
Dvl (or Dsh)	Disheveled	Inactivates GSK3β
β-catenin		Stimulates transcription when complexed with TCF/LEF-1
TCF (variants) LEF-1	T Cell-specific Factor Lymphoid Enhancer-binding Factor 1	Stimulate transcription when complexed with β-catenin
Roles in repression of Wnt target genes		
GSK3β CK1	Glycogen Synthase Kinase 3β Caseine Kinase 1	Phosphorylate β-catenin
APC Axin	Adenomatous Polyposis Coli	Facilitate β-catenin phosporylation
β-TrCP	β-Transducin repeat-Containing Protein	Ubiquitinates β-catenin
Groucho		Represses transcription by binding to TCF/LEF-1
Dkk-1	Dickkopf	Binds to LRP, paninhibitor of canonical pathway
sFRP	Secreted Fzd-Related Peptides	Sequesters Wnt protein
WIF-1	Wnt Inhibitory Factor-1	Sequesters Wnt protein

1.5.2 Effect of canonical Wnt signalling on hematopoietic stem cells

Wnt ligands are produced by both hematopoietic and microenvironmental cells [147, 148]. Many studies have followed up initial reports indicating that Wnt-signalling has a mitogenic effect in HSCs/early progenitor cells [147, 148]. Although somewhat controversial, Wnt signalling is believed to contribute to HSC self-renewal. The canonical pathway has been experimentally stimulated in cultivated HSCs and has in some settings resulted in elevated cell pools as judged by phenotype and function (reconstitution potential) [137, 149, 150]. Pathway triggering was done either by Wnt-exposure or by

forcing β-catenin expression ("gain of function") using retroviral vectors. However, similar *in vivo* manipulations of HSCs have resulted in fatal effects [151, 152]. This outcome may have been caused by a β-catenin "overdose", urging the need for fine tuning of the pathway. Accordingly, an opposite strategy, deleting β-catenin in HSCs ("loss of function") was shown to impair stem cell renewal [153]. Another deletion strategy has recently demonstrated a vital role for Wnt3A in stem cell renewal during fetal hematopoiesis [154].

Confusingly, there are conflicting reports. Normal *in vivo* hematopoiesis has been recorded even after disabling β-catenin in HSCs [155]. Moreover, similar results were obtained from deletion of both β-catenin and its homolog γ-catenin (also known as plakoglobin), excluding the possibility of catenin redundancy [156]. Yet, a role for the canonical pathway may apply after all, as an unidentified Wnt signalling tranducer has been suggested. Using a reporter assay, HSCs were shown to convey canonical Wnt signals into transcription in the combined absence of the known catenins, and these HSCs were found to function normally [157].

The importance of Wnt signalling in stem cell renewal has been confirmed by an approach acknowledging the potency of the microenvironment. Osteoblasts were manipulated to secrete Dkk-1 (canonical pan-inhibitor) *in vivo*. Whereas hematopoietic cell numbers were close to normal, this way of blocking the Wnt signal caused HSCs to suffer from a loss of function (impaired reconstitution potential) [158].

It has been suggested that Wnt does not primarily stimulate replication, but instead control dedifferentiation, thereby controlling a reflux process that may sustain stem cell self-renewal and differentiation potential [159]. Additionally, Wnt can act on hematopoietic cell through non-canonical pathways [143].

1.5.3 Effect of canonical Wnt signalling on B-cell progenitors

Besides an influence on HSC renewal, the canonical Wnt pathway may have a role in HSC commitment and fate of early hematopoietic progenitor cells.

Reconstitution experiments using Wnt3a-treated murine HSCs indicated a bias toward B-lymphopoiesis [137, 149] and the first experiments on B-lineage cells coincided with the contemporary consensus that canonical Wnts in general lead to proliferation of stem/progenitor cells. When pro B-cells from a fetal mouse were exposed to Wnt3a in culture, they were found to proliferate through a LEF-1 dependent mechanism (i.e. Wnt

pathway) [160]. Accordingly, abnormal B-lymphopoiesis was observed in mice lacking LEF-1 or Fzd 9, and this phenotype was interpreted as a consequence of an impaired Wnt response [160, 161]. Of note, effects of LEF-1 absence does not necessarily relate to an impaired Wnt signalling, since LEF-1 have Wnt-independent functions. These are based on LEF-1 interaction with co-activators other than β-catenin [162] and its repressor activity when paired with Groucho. Later, a more differentiated picture has evolved regarding the role of Wnt in B-lymphopoiesis. A murine study found that Wnt3a reduced B-lymphopoiesis, but only when the cultures were supported by a stromal feeder layer. Hence, a negative Wnt effect appeared to be mediated via the stromal cells [163]. A stroma-based approach was also used in a later study, providing evidence that distinct Wnt ligands regulate the early events in murine hematopoiesis differently. Expression of Wnt family proteins was forced in stromal cells and Wnt3a was found to inhibit B-lymophopoiesis while Wnt5a stimulated this process [164]. Wnt5a acts through a non-canonical pathway and has previously been reported to oppose canonical signalling [165] and to suppress B-lymphopoiesis in a fetal system [166].

The role of canonical Wnt signalling in malignant pre B-cells is also controversial, as opposite effects on survival and proliferation are reported [167-169].

1.6 Use of microfabricated tools for cellular studies

For over a century, cell culture technology has essentially been synonymous with growing cells on a large, planar and homogeneous surface immersed in a homogeneous fluid medium. Clearly, the inability to grow stem cells under conditions mimicking their native habitat (complex and three-dimensional) hampers both their clinical potential and the physiological relevance of *in vitro* stem cell research.

Microfabrication technology enables manufacturing of components and devices with micrometer resolution and creates new opportunities for experimental research [170-173]. Culture systems that are more *in vivo* -like can be produced, both with respect to geometry (topographical structures [174, 175]) and protein surfaces (substrate patterning [176]). Also, patterned co-cultures for the study of cell-cell interactions have been achieved [177]. Importantly, by controlling the fluid flow in the microscale environment it is possible to regulate transport of fluids and soluble factors (microfluidic delivery). In particular, the phenomenon known as laminar flow allows functionalities not achievable in macrosystems. Because there is no turbulence in a microchannel, the fluid flows in

parallel layers and will not mix except by diffusion. This flow pattern enables a differential stimulation of a culture, e.g. a given stimulus can be administered in one or several specific layers. Using this principle, microfluidic cell cultures have been stimulated focally [178, 179] or by a stable gradient [180]. Gradients of signalling molecules are crucial for the establishment of polarities in stem cell niches *in vivo* [181-183]. The ability to mimic this signalling characteristic may contribute importantly to the imitation of stem cell niches in the laboratory and thus represents a new tool for stem cell research. However, microfluidic conditions are very different from those provided by classical cultivation, as they involve factors like shear stress and high Surface Area to Volume ratios, SAVs [184, 185]. Thus, the study design must be customised accordingly.

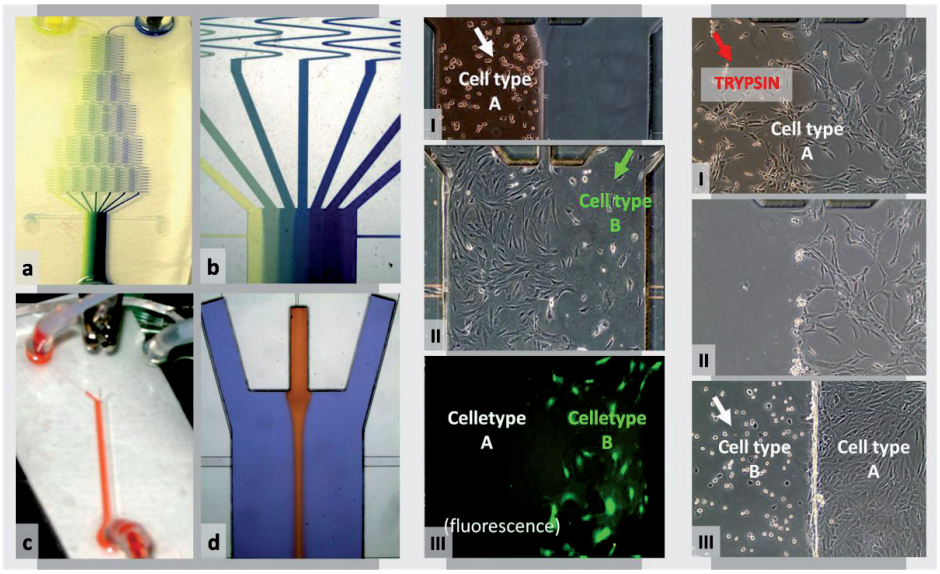


Figure 7 Laminar flows in microchannels can be used for making cultures that are heterogeneous with respect to chemical environment and/or cellular composition. Left panel: dyed solutions demonstrating laminar flows in a cell-free microsystem. The cell chambers receive a gradient (a, b) or focal streams (c, d). Middle panel: use of laminar flow for sequential seeding of distinct stromal cells at different locations (middle and lower picture show the same culture imaged with phase contrast and fluorescence respectively). Right panel: use of laminar flow for localised trypsination and re-seeding. The trypsination regime also enables the culture to be harvested/analyzed at different time intervals during an experiment. See also **Figure S1**, page 61.

1.7 Advanced methods

This study applied two methods not mainstream in stem cell research; a signal sequence trap (SST) for identification of cell surface and secreted proteins (Article I) and soft lithography to produce microsystems for cell cultivation (Article IV). Below is a glance at these methods, providing concepts and some details.

1.7.1 Signal Sequence Trap by Retroviral EXpression (SST-REX)

Membrane-bound and secreted proteins have a hydrophobic signal peptide in their N-terminal region. This "label" serves to direct the proteins through the secretory pathway (involving endoplasmatic reticulum, Golgi apparatus and vesicles) before they are launched to the periphery. As indicated by their name, SST methods are designed to identify such proteins. The methods are based on a cDNA library, which contains complementary DNA molecules synthesised from mRNA molecules in a cell population, thus reflecting those genes being expressed at the harvesting time.

We employed a variant called **S**ignal **S**equence **T**rap by **R**etroviral **EX**pression, SST-REX, **Figure 8**, which is based on a retroviral vector for cDNA library construction, pMX-SST [186]. Downstream to the vector's cDNA insertion site is a segment encoding a cytokine receptor that is constitutively active (without ligand binding), but importantly, its signal sequence is missing. Thus, only those cDNAs containing a signal sequence, in reading frame with the receptor, allow for a translated product that can reach the cell surface and be functional. Screening for such cDNAs is done by expressing a retroviral library in a cytokine-dependent cell line at cytokine-free conditions. The cDNAs containing a signal sequence will rescue their host cells and can be harvested from growing clones. Finally, they are identified by PCR-amplification, sequencing and comparison with reported sequences in public databases.

Technically, the construction and expression of the retroviral cDNA library was enabled by 1) electroporation of the plasmid cDNA library into competent bacteria for amplification, 2) transfecting of the plasmid cDNA library into a virus packing cell line (Phoenix Eco) for virus production, and 3) use of the virus containing supernatants for infection of the screening cell line (Ba/F3). Parallel control experiments were enabled by control vectors, expressing green fluorescent protein (pMX-GFP), the cytokine receptor in frame with a signal sequence (pMX-SP) or a corresponding variant devoid of a signal sequence (pMX-ΔSP).

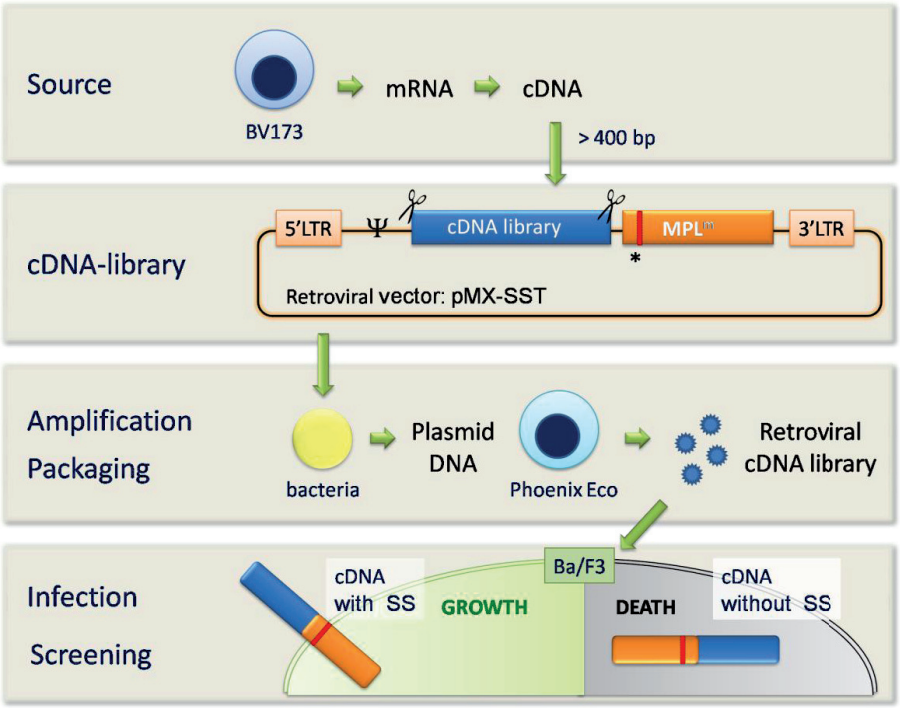


Figure 8 Schematic drawing of SST-REX procedure. **MPLm**: truncated thrombopoietin receptor, *: activating mutation in transmembrane domain. **Scissors** indicate BstX1 insertion sites**, > 400 bp** indicate a purification step of cDNA fragments longer than 400 base pairs (using a SizeSep 400 spin column). **SS**: signal sequence.

1.7.2 Soft lithography, photolithography and bonding

Soft lithography is a convenient, effective and low-cost method for manufacturing of micro and nanostructures in an elastomeric ("soft") material, see **Figure 9** and a schematic outline of the procedure in section 6.2, page 66. It was developed in the late 90ies and can be used for applications additional to what is described here [187]. The method is based on photolithography, a technique originally used to fabricate the integrated circuits inside computers. The result of the photolithographic process is a relief pattern, called master, or master mold. This pattern is inversely replicated in a silicon rubber; Poly Di Methyl Siloxane (PDMS) in a process called replica molding.

Such a PDMS replica can be used for different purposes, such as a stamp for printing of micropatterns [176]. Alternatively, the PDMS replica can be bonded to a flat substrate to realise a microsystem [188]. As evident from Article IV, a microsystem of PDMS microchannels bonded to polystyrene (PS) serves as an appropriate platform for

microfluidic cell cultures. The steps used for fabrication of such devices are described briefly in order to inform a general life science researcher, being unfamiliar with microfabrication.

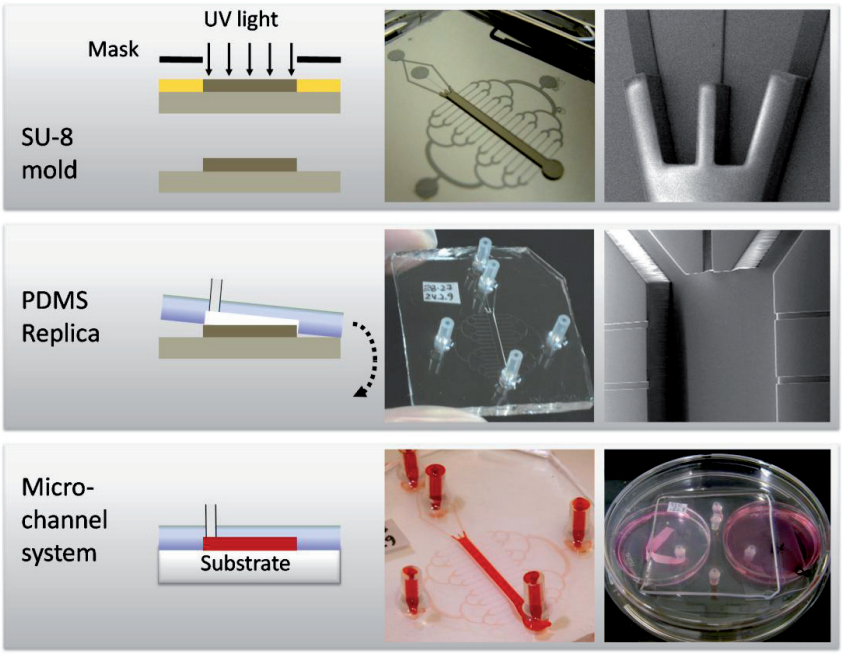


Figure 9 Mold, replica and microchannel system; Schematic cross-sections and images. The width of the main channel is 1,5 mm.

In **photolithography**, a photosensitive material (photoresist) is spun on a thin disk of silicon (wafer), serving as a carrier. The photoresist is then exposed to light trough a mask, thereby changing its properties in illuminated areas. Depending on the choice of photoresist, the light will either enable polymerisation of the photoresist (applies to resists known as *negative*) or make it prone to dissolvement (applies to photoresists known as *positive*). Accordingly, treatment of the partly exposed photoresist with a developer results in selective removal of material, and the desired features are left behind. Thus, the resulting pattern is either a positive or negative copy of the mask used for illumination. Photolithography is performed in a cleanroom with appropriate lightening, as light and particles will destroy the microstructure being produced. The processing is conducted within a fume hood, due to the volatile nature of unprocessed photoresists and their developers.

SU-8 photolithography Masters for replica molding are commonly made by SU-8 photolithography. SU-8 is a negative, epoxy-based photoresist, **Figure 10**. It has low optical absorption, allowing light to readily penetrate the material. Thus, it is possible to produce "thick" (0,5 to > 0, 25 mm) layers with near vertical side walls. SU-8 is supplied in different formulations, each optimised for a certain range in layer thickness. Plastic transparencies of high resolution are used as masks. The processing steps are given in **Box 7** below, based on own experience and guidelines provided by the manufacturer (MicroChem Corp, US). The final features of the pattern are evaluated by a microscope and a profilometer (measuring thicknesses).

PDMS replica molding PDMS is a silicon-based organic polymer which has properties amenable for fabrication of microscale cell culture chambers, see **Box 8** below. The variant used in soft lithography (Sylgard 184, Dow Corning) is supplied in two components; a curing agent and a base. They are mixed together in a 1:10 ratio (weight/weight) and poured onto the master. The liquid pre-polymer conforms to the shape of the master with high fidelity. Bubbles formed by the mixing are removed by degassing in a vacuum chamber. Curing occurs when vinyl groups ($-CH=CH_2$) present in the base react with silicon hydride (S-H) groups in the curing agent to form a cross-linked solid, **Figure 11**. This reaction is accelerated in an oven (65°C, overnight). The cured polymer is very elastic and has low surface energy due to the highly flexible siloxane backbone and low forces between the methyl groups (Dow Corning). These properties enable the cured PDMS replica to be removed from the master without harm to either party, thus allowing the master to be reused. Yet, the release is facilitated by coating the master mold surface prior to the first molding with a fluoro-containing silane (trichloro(1H, 1H, 2H, 2H-perfluorooctyl)-silane).

Bonding of PDMS replica to substrate PDMS seals spontaneously and reversibly to most flat surfaces by van der Waals forces. This bonding is watertight, but cannot withstand pressures greater than ~5 psi [188]. Permanent bonding to glass or another PDMS surface can be obtained by exposing the two surfaces to oxygen plasma and bring them in conformal contact. The plasma treatment is thought to introduce silanol groups (Si-OH) at the expense of methyl groups (Si-CH3). This modification both makes the PDMS surface hydrophilic and able to make covalent Si-O-Si bonds with another surface in a condensing reaction. The plasma treatment also serves to sterilise the materials [190].

Box 7 SU-8 Processing

Wafer pre-treatment. This step ensures good adhesion between the photoresist and the wafer. The wafer may be cleaned (by chemical solutions or plasma, not crucial when applying new wafers). Just prior to use, moisture is eliminated from the wafer surface by heating.

Photoresist application and coating. SU-8 photoresist is manually dispensed onto the wafer. Due to high viscosity, care must be taken to avoid air bubbles. By the use of a spinning device (spin coater), the resist is subjected to two spin cycles; the first cycle serves to spread the resist uniformly onto the whole wafer surface, and the final cycle defines the final thickness of the resist layer. (Actually, this parameter depends on the amount of resist applied as well as the spinner settings).

Pre-baking. Heat is used for evaporation of solvent in the photoresist, making it denser. A hot plate is preferred, as a convection oven will cause a “crust" on the SU-8 surface, reducing further evaporation. A two-step procedure (with a lower initial bake temperature) allows the solvent to evaporate at a more controlled rate.

Masking and Exposure. The appropriate wavelength (365 nm) is provided by an illuminating instrument (“photo-aligner”) and results in formation of a strong acid in illuminated areas. The optimal exposure dose depends on the wafer material (its reflective properties) and the thickness of the resist layer.

Post-baking. Initiated by the acid (produced by the exposure step), the elevated temperature results in selective cross-linking of the resist in illuminated areas.

Development. The wafer is submerged in the developer (1-Methoxy-2-propanol acetate), making the non-illuminated SU-8 dissolve into solution. Actual dissolution rates can vary widely as a function of agitation rate, temperature and resist processing parameters.

Other remarks The wafer is kept levelled at all times, as gravity can affect the flatness of the SU-8 layer. Optimum cross-link density is realised by adjustments of the exposure and baking regimes. For instance, a low UV dosage will provide insufficient amounts the catalytic acid, resulting in slow cross-linking. After the baking steps, the resist needs to cool slowly to room temperature before further processing. This thermal relaxation reduces internal stress and is crucial for survival of the mold [189]

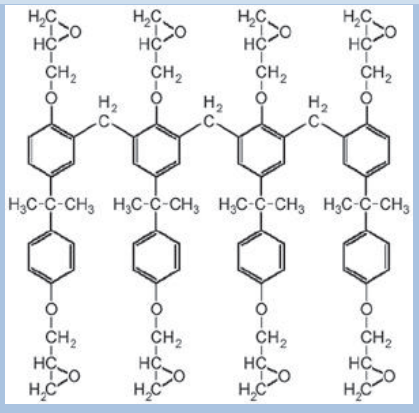


Figure 10 SU-8 polymer Drawing from Technical University of Denmark.

Box 8 PDMS; properties and cross-linking process

- Conforms to submicron features
- Gas permeable
- Biocompatible and non-toxic
- Transparent
- Non-fluorescent
- Stable, chemically and thermally
- Easily bonded
  - Spontaneous and reversible to many surfaces,
  - Induced and irreversible to itself, glass or silicon nitride
- The hydrophobic surface can be made hydrophilic by exposure to oxygen plasma

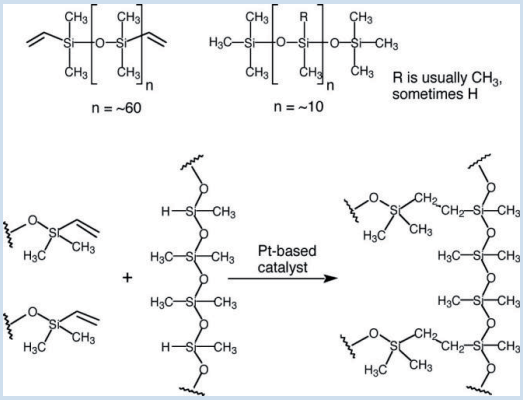


Figure 11 PDMS crosslinking Drawing from University of Wisconsin.

2 Aims of the study

The hematopoiesis process is an example of the dynamic regulation of stem/progenitor cells by the microenvironment. According to the changing needs of the organism, the BM microenvironment tunes self-renewal as well as differentiation of HSCs. In this way the stem cell pool is maintained while all the specialised cells found in the blood are generated.

Early B-lymphopoiesis provides an experimental model for understanding the communication between stem/progenitor cells and the microenvironment for several reasons:

- This process depends critically on the microenvironment,
- It can be imitated *in vitro*, notably in the presence of microenvironmental supportive cells, i.e. stromal cells of various origins,
- The primitive HSCs and the different B-cell maturation stages can be identified by a broad spectrum of markers,
- The role of different stromal phenotypes can be tested, including the use of mesenchymal stem cells that can be controllably differentiated.

This study was designed to explore the hematopoietic environment that controls early B-lymphopoiesis by:

1) Search for proteins displayed by human B-cell progenitors that may mediate their communication with the microenvironment (Article I).
2) Determine the localisation of B-cell progenitors in human BM and search for concomitant stromal factors (Article II).
3) Develop cultivation methods for analysing human B-lymphopoiesis *in vitro* and investigate whether this process is affected by triggering of the canonical Wnt pathway (Article III).
4) Develop a microfluidic system for long-term cell cultivation with the prospects of making more advanced models of stem cell niches in the BM (Article IV).

3 Summary of articles

Article I

Characterization of the novel human transmembrane protein 9 (TMEM9) that localises to lysosomes and late endosomes.

We implemented the SST-REX method as we wanted a systematic approach to search for candidates that may contribute to the B-cell progenitor-stromal interaction. This method is used for cloning secreted and membrane bound proteins of a given cell population. The leukemia cell line BV173 was chosen as a representative B-progenitor cell, based phenotype and ability to interact with stromal cells in culture, both physically and functionally.

One novel clone was selected for further characterisation, based on the nature of its potential open reading frame, indicating a transmembrane type 1 protein. Northern blot analysis revealed a wide-range expression in different tissues. Likewise, expression was demonstrated in multiple hematopoietic cell lines of B, T, myeloid and erytrhoid origin, with no correlation to maturation level. The putative protein coding part (PCR-amplified from another cDNA library) was transfected into COS-1 cells for translation. Western blot characterisation revealed a 26 kilodalton core product and 3 possible glycosylation variants. Immunohistochemical studies indicated a cellular localisation within "permanent" cytosolic membranes, a position that is also defined by a signal peptide. This localisation indicated a function in intracellular transport and precluded an obvious role in intercellular communication. The protein was named transmembrane protein 9 (TMEM9).

Article II

$CD10^+$ stromal cells form B-lymphocyte maturation niches in the human bone marrow.

We wanted to display the distribution of B-cell progenitors in human BM and investigate whether their interaction with stromal cells could be visualised. Numerous sample target cells were ensured by selecting biopsies from patients with high rates of normal lymphopoiesis (i.e. non-specific reactive BM). The benign nature of the samples was

confirmed by the absence of clonal clusters and a normal phenotypic distribution (Pax 5^+, $CD20^+$, $CD34^+$, TdT^+ cells).

Pax-5 immunolabelling enabled visualisation of the total B-lineage population, demonstrating scattered cells throughout the BM tissue, with no relation to bone fragments. A closer inspection revealed a non-random distribution of B-cell progenitors at the cellular scale; the majority of Pax-5^+ cells were organised in rows, suggesting a linear regulatory element.

Two-colour immunohistochemistry disclosed that all B-lineage cells was in contact with slender $CD10^+$ $VCAM^{+/-}$ cells (body or extensions), indicating an essential role of this stromal phenotype. Furthermore, the use of double and triple immunoassays demonstrated that B-cell maturation was oriented towards the sinusoids, appropriately for loading the end product into circulation.

About 50% of the hematopoietic $CD34^+$ population co-localised with the $CD10^+$ stromal population, a reasonable finding due to their fraction of stroma-independent cells, i.e. committed progenitors of non-B-lineages. Similarly, only random association with $CD10^+$ stromal cells applied for other cells not depending on stromal contact, i.e. developing red cells and myeloid cells, as well as infiltrating B-cells (malignant or benign).

In conclusion, this was the first evidence that human BM B-cell progenitors receive specific support from a $CD10^+$ population of stromal cells and that B-cell maturation proceeds in well-organised directional arrangements towards the sinusoids.

Article III

Wnt expression and canonical Wnt signalling in human bone marrow B lymphopoiesis.

We wanted to examine whether B-progenitor and stromal cells in the human BM harbour the molecular machinery necessary to drive the canonical Wnt pathway. Finding this premise fulfilled, we further investigated how triggering of this pathway affected early B-lymphopoiesis.

RT-PCR analysis revealed that B-cell progenitors express mRNA encoding central Wnt pathway proteins: i.e. multiple Wnt receptors (Fzd), and co-receptors (LRP5 and

LRP6), the key intracellular signalling molecule (β-catenin) and its homolog (γ-catenin[24]), and the nuclear activators (LEF-1 and TCF7L2[25]) that induce transcription of Wnt target genes. The screening also detected mRNA coding for pathway antagonists (sFRP, WIF1, Dkk) as well as Wnt ligands. Some of these mRNA species were quantitated by Real-time PCR and showed distinct expression levels in B-cell progenitors at different maturation stages. These results suggest that canonical Wnt signalling regulate early B-lymphopoiesis and indicate both autocrine loops and fine-tuning of the pathway by antagonists. However, a complex picture emerged, as this pathway is also active in BM stromal cells.

The presence of a functional Wnt pathway was confirmed by Western blotting, demonstrating stabilisation of β-catenin in B-cell progenitors after short-term exposure to Wnt3a in culture. The long-term consequence of pathway triggering was tested by two different cultivation assays, both depending on stromal feeder layers for survival of hematopoietic cells. The B-lymphopoiesis assay demonstrated that Wnt3a reduced the production of B-lineage cells from HSCs ($CD133^+CD10^-$ population). The B-progenitor maintenance assay demonstrated that Wnt3a lowered the number of rescuable B-progenitor cells ($CD10^+$) after 2 weeks in culture, an outcome explained by a slowed cell proliferation (measured by CFSE tracking).

Due to the presence of Wnt-responsive stromal cells in both assays we concluded that canonical Wnt signalling acts directly or indirectly to repress human early B-lymphopoiesis *in vitro.*

Article IV

Extensive adipogenic and osteogenic differentiation of patterned human mesenchymal stem cells in a microfluidic device.

In preparation towards fabrication of stem-cell niche models on-chip, we wanted to develop a suitable microscale system. Thus, we used soft lithography for fabricating a microfluidic platform reported to comply with long-term cell cultivation. As MSCs and their progeny can support stroma-dependent hematopoietic cells, MSC differentiation was chosen as a relevant test process for evaluating whether the microscale conditions were in accordance with normal cell behaviour.

[24] γ-catenin is denoted plakoglobin in article III
[25] TCF/L2 is denoted TCF-4 in article III

The initial system was composed of PDMS microchannels bonded to a glass substrate and did not allow for consistent MSC differentiation. This anomaly was possibly due to cells spreading out in the chip, thereby obstructing the channels used for medium supply. Hence, cell spreading was prohibited by making the perfusion areas non-supportive to cell growth. A new and convenient patterning method based on a polystyrene substrate (PS) was developed for this purpose and showed to be compatible with MSC differentiation. In the revised microsystems, on-chip differentiation of MSCs to adipocytes and osteoblasts was superior to previous documentation and correlated with results obtained by traditional cultivation. The change from a glass substrate to a polystyrene substrate may have contributed to a better microscale environment. The revised systems were operated by a size-effective "flip-chip" set-up that compensated for the less robust PDMS/PS bonding (compared to PDMS/glass).

In conclusion, the reported system shows compatibility with normal cell behaviour on a long-term basis. The system allows for unique manipulation of the culture by the use of laminar flow and is a candidate platform for fabrication of complex models of hematopoietic niches and other tissue engineering purposes.

The article was featured as the main story on the journal's homepage (in April) and was reviewed online (on 29th Mars 2010) by "Highlights in Chemical Biology", which is another publication by the same publisher (Royal Society of Chemistry), see section 6.3, page 67. Artwork from this article will be featured on the "Lab on Chip" inside front cover in May 2010 (the preliminary version is placed in front of Article IV).

4 Discussion

4.1 Why study stem/progenitor cells and their microenvironment?

Research on stem cells and early progenitor cells seeks to provide basic understanding of how cell behaviour is regulated, both in embryogenesis and in tissue maintenance. Such knowledge may ultimately lead to clinical control of tissue regeneration. Additionally, stem cell research is of utmost importance in order to understand and fight tumourigenesis. The cancer stem cell concept has gained strong focus, implying that cancer may originate from cancer stem cells, which are dysfunctional stem cells and/or malignant cells that have acquired stem cell properties. This understanding explains how cancer cells can escape current therapies [191].

These research areas converge in the need for identifying the microenvironmental elements and mechanisms that dynamically regulate stem cell behaviour. Conceivably, aplasia (insufficient production of new cells for replenishment) may be the result of a non-functional microenvironment, and cancer the consequence of aberrant stem cell activity outside of their normal environment [192]. Although the complex interactions between stem cells and the microenvironment are far from determined, similarities between embryogenesis, adult stem cell differentiation and cancer development can be noted as these processes are dictated by several of the same genetic pathways, e.g. BMP, TGF-β, Wnt, fibroblastic growth factor (FGF), Notch and Sonic Hedgehog Homolog (SHH)-pathways [193].

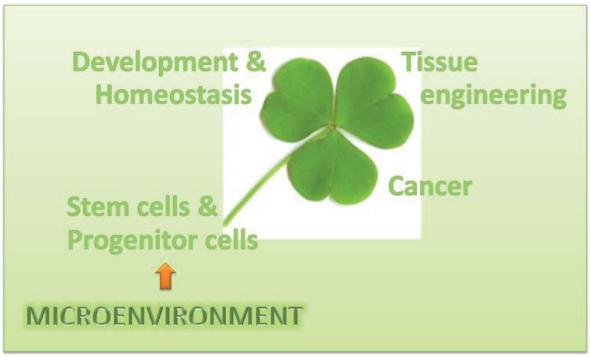


Figure 12 Relevance Research on stem/progenitor cells and their regulation by microenvironmental cues is relevant in multiple research branches.

4.2 Methodological considerations

4.2.1 "Evolution" in methodology

In science (and in general), some methods can hardly be imagined to come out of date, e.g. microscopy. Others are replaced by strategies being superior in various ways, due to informational value, safety, cost & time consumption. Additionally, new methods are adopted in parallel to conventional variants. Such an "evolution" in methodology is evident from the methods used in this study. Regarding gene screening, the signal sequence trap methods have stepped aside in favour to more rational gene profiling methods using microarrays. In contrast, use of microfabricated tools for manipulation of cell behaviour is a field likely to progress in the future.

4.2.2 Implementation of the SST-REX methodology

Motivation Signal sequence traps (SST) serve to identify extracellular proteins based on their characteristic signal sequence. The SST-REX variant was reported as more efficient than previous SST methods, due to incorporation of longer cDNA fragments and a sensitive functional selection (a single step selection for factor-independent growth) [186]. Long cDNA fragments ensure safe gene identification and reduce the risk of random hydrophobic sequences operating as "false" signal sequences. These capabilities and access to a new virus laboratory at DNR encouraged us to implement SST-REX and replace a previous, non-viral variant [194].

The implementation of this methodology was an interesting challenge due to its many facets, **Figure 8**, page 26. Molecular engineering techniques were needed to prepare the initial plasmid library and identify the finial screening products. Intermediate cultivation steps, using bacterial and mammalian cells respectively, were needed to 1) amplify the plasmid library, 2) transform it into a retroviral library and 3) perform the growth based screening. These cultivation steps coincided with three different principles for introducing nucleic material into cells; 1) electropolation, 2) transfection and 3) infection.

Outcome Obviously, a successful outcome of a multistep procedure depends on proper performance of each single step. Construction of cDNA libraries is a demanding task, but was mastered by other members at our lab. Aided by their experiences I produced an adequate BV173 library (containing 1,4 x 10^6 cfu/µg DNA with an average

insert size of 750 base pairs). However, we had no experience with eukaryotic retroviral expression. It turned out that I needed to optimise all steps; hence implementation of the total procedure seized one full year's work. Basically, it was necessary to eliminate a range of non-effective materials **Table 4**. In addition, a variant infection procedure was implemented, involving cell centrifugation during infection, as opposed to regular static conditions **Figure 13**.

Table 4 Optimisation of SST-REX procedure.

Parameter	Original regime / Problem	New regime / Benefit
Packaging cell line	• Bosc 23 Unstable genotype; A low cell fraction keep the genes necessary for virus packaging	• Phoenix Eco More stable genotype, Incorporation of viral genes can be monitored by flow cytometry
Transfection agent	• Calcium phosphate, or • Lipofectamin Low efficiency;* ⇨ viral titer = 0,1 x 10^6	• Fugene High efficiency;* ⇨ viral titer = 0,9 x 10^6
BaF3 cell line stock	• Donated Mycoplasma infected ⇨ Infection resistant	• Quality controlled (DSMZ**) Mycoplasma free ⇨ Susceptible to infection
Infection procedure	• Static ⇨ Low infection efficiency	• Spin infection ⇨ High infection efficiency

* Viral titer depends on both transfection efficiency and on the cells' health.
** German collection of microorganisms and cell cultures.

The spinning procedure does not sediment viral particles, but *"it is thought that virus on membrane fragments is spun onto cells in a manner which effects greater infection"* (G. P. Nolan, Stanford University, provider of Phoenix packaging cells). The spin-infection efficiency was found to depend on the G-force, time and the cell/virus ratio, **Figure 13**. In contrast, repeated additions of viral supernatant had negligible effect (not shown). For the final infection of the retroviral library we used 4 cells/virus and 500g for 1h, resulting in 10% infection efficiency. This regime resulted in multiple viral integrations in 11% of the surviving BaF3 clones, which was acceptable. (In contrast, 33% of the clones had

multiple integrations when using 1000g for 2h and same cell/virus ratio). Clones with multiple integrations were discarded as they preclude identification of the cDNA insert responsible for BaF3 survival.

Proteins identified by SST-REX methodology are presented in **Table 6**, page 46. Additional "false" clones had inserts that were out of reading frame with the cytokine receptor, an inverted cDNA insert (3' to 5'), or lacked a signal sequence.

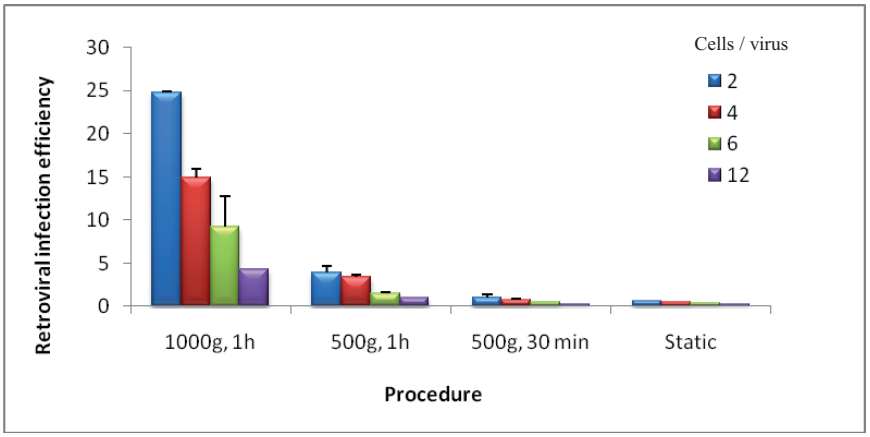


Figure 13 Infection procedures Conditions tested for adjusting the percentage of BaF3 cells infected with a retroviral library. Four cell/virus ratios were tested (see label) at static conditions and three different cell centrifugation regimes (variable G-force and time, as indicated). Spin-infected samples were run in duplicate. Error bars indicate standard deviation (SD).

Perspective In spite of efficient microarray analysis, there are still recent reports based on SST-REX screening for identifying cell surface molecules [195, 196]. Methodological improvements include the use of distinct insert sites for cDNA integration into the pMX-SST vector, ensuring a directional cloning. Also, the BaF3 clones are passaged several times before clone rescuing, a strategy likely to reduce the number of false positives [195]. Unlike other gene analysis techniques, SST-REX can be used for obtaining both genetic information and recombinant proteins simultaneously. Because post-translation modifications of a mammalian type are preserved (involving sugar and fatty acid chains), this method is well-suited for development of monoclonal antibodies by using a BaF3 clone as immunogen.[26] Likewise, the eukaryotic expression system has potential for improving the readout of SEREX technology (serological identification of tumour

[26] SST-REX is core technology for the company named ACTGen (actgen.co.jp)

associated antigens by recombinant expression cloning), by allowing for identification of postranslationally modified antigens [197].

4.2.3 Hematopoietic assays

Cell source Functional studies on early B-cell lymphopoiesis are mainly performed in the murine system. This is not only explained by the fact that human cells are more troublesome to provide, but also reflects the experience that human B-cell progenitors are more demanding to cultivate (see section 1.4.2, page 16). By focusing our research on primary cells of human origin, we went for the gold standard in studies aiming for therapeutic translation. Arguments against two other obvious options are:

- Continuous cell lines are valuable research tools, but unfortunately they do not always recapitulate the behaviour of their cognate primary cells. This deviation may be due to their lack of growth control and/or genetic drift over time.

- Murine systems have provided important basis for our general understanding of hematopoiesis and allows for flexible experimental *in vivo* designs. However, significant differences between species cannot be ruled out.

The reviewer of Article III acknowledged our choice, stating "*It is important that more differentiation studies are done in human systems, as this is the only way to ensure that basic knowledge is applied to clinical problems*"

Cell management *ex vivo* The value of primary cells in functional assays depends on whether we can maintain them in a representative state, both during isolation and subsequently during cultivation. Isolation of primary hematopoietic subsets is time-demanding, as the initial harvesting step is followed by several types of purification. We routinely used combinations of a) density gradient centrifugation, b) Dynabeads®-based isolation c) magnetic cell sorting (MACS) and fluorescence activated cell sorting (FACS). Such multistep procedures are likely to stress the cells and induce apoptosis. Our preventative measures to limit cell stress included the use of a) buffered media, for keeping cells nourished and at pH 7.4, b) low temperature when applicable, to minimise cell metabolism, and c) protein supplements for physical stabilisation (bovine serum albumin, BSA or fetal calf serum, FCS).

Having the cells successfully isolated, the next challenge is to provide them with a proper environment in culture, which is a problem of the "hen and egg" category.

Information on their requirements in the body may guide us in optimising the culture conditions. Vice versa, factors necessary for survival in culture indicate their demands in native environments. Moreover, the optimal conditions may depend on the cell maturation level. Whereas experiments on B-cell subsets must meet with their stage-specific requirements, investigation on the earliest differentiation process must also accommodate the multilineage precursors.

Based on the literature, we implemented a method for co-cultivating HSC on a pre-seeded layer of MS-5 cells, **Figure 15** (page 45, right panel). This murine cell line was reported to favour HSC differentiation towards the B-lineage cells [109]. MS-5 was also kept as a standard feeder layer for maintenance of B-cell progenitors, due to more reproducible results than obtained by another murine alternative (S17 cell line). Occasionally, primary human stromal cells were used for comparison. Later, the development of an iMSC cell line (iMSC#3, Ola Myklebost and co-workers) equipped us a stromal cell line of human origin. The MSC population is an important candidate for contribution to the hematopoietic microenvironent (see page 50).

We performed several tests for evaluating the role of each assay component. Our aim was to determine a set of parameters giving "base-line" readout (i.e. $CD19^+$ cell number), thus allowing us to detect a potential effect of any given agent. Regarding the B-lymphopoiesis assay, we tested several medium compositions, with variant serum concentrations and cytokine combinations and dosages (SCF, G-CSF, IL-3). The final assay included the same components as previously described [109], but we reduced the concentrations of supplements. In contrast to the use of $CD34^+$ cells as a seeding population [109], we seeded $CD133^+$ hematopoietic cells (HSC/progenitor cells), thereby avoiding pro B-cells (being $CD34^+$)

Parameters in functional assays Cell behaviour is directed by signalling pathways that are complex by themselves and further complexity is added by their interactions with other pathways in regulatory networks (e.g. Wnt signalling). Thus, a number of non-intended influences may direct the functional readout of a given test agent. Especially when using complex models, it is challenging to control all parameters. Our cultures of hematopoietic cells included two obvious non-defined components; FCS (at reduced amounts) and stromal cells, which may both lead to biased cell responses.

Results showing similar trends indicate that experiments are reproducibly performed. Nevertheless, it is important to remember that they are a product of the

experimental test system. Hence, caution about their physiological relevance is necessary. Furthermore, when evaluating observations made by different research groups, one should consider the experimental design, including the following parameters:

- Source of test-agent (e.g. conditioned media or recombinant molecule)
- Cell source (species, tissue type & developmental stage, cell maturation level),
- Medium components and
- Timing.

The significance of ontogeny is emphasised by a recent study, showing that IL-7 is essential to B-cell progenitors developed from BM progenitors, but not to those originated from cord blood (CB) progenitors [119].

Precision This study dealt with low cell numbers, both in cultivation assays as well as in Real-time PCR analysis. Hence, robust quantitation methods were required. Regarding *cell quantitation by flow cytometry*, precise cell counts were obtained by adding a known number of beads to the samples, see section 6.1.3, page 65. A similar strategy has later been termed single bead-enhanced cytofluorimetry (SBEC) [198]. Regarding *PCR-quantitation*, much work was put in by Guri Døsen to determine a suitable endogeneous control (i.e. phosphoglycerate kinase 1, PGK1), allowing for comparison of different samples. Normalisation is necessary due to variability between samples, caused by different concentrations/degradation of mRNA and variable efficiencies during cDNA synthesis and PCR.

4.2.4 Fabrication of microfluidic devices & substrate modifications

The soft lithographic method is well described in the literature and was implemented without major technical difficulties. The process parameters were slightly adjusted to meet with the equipment at our laboratory. Given precise performance of each step, functional microfluidic devices were produced, **Figure 9**, page 27).

In contrast, a reliable definition of cell-supportive and non-supportive areas within the devices was more demanding to obtain. The literature describes several cell patterning techniques, but these methods are typically time-consuming and some involve the use of hazardous chemicals [199]. Thus, I explored more convenient and safe strategies, as summarised in **Table 5** below. Reversibly bonded PDMS masks were used for selective treatment of exposed areas. The performance of the surface-modified substrates was tested by conventional cell cultivation, using the cell line iMSC#3.

Using approach A, a FN solution was drawn into PDMS microchannels for deposition on the glass surface, followed by mask removal and BSA blocking of the remaining area (result shown in **Figure S2**, page 62).

The other approaches were based on the use of Pluronic as a blocking agent and depended on pre-prepared hydrophobic areas, a prerequisite for Pluronic adsorption, see **Box 9**. For this purpose, approach B and C used selective deposition or removal of chloro or fluoro-containing silans (allyltrichlorosilane or trichloro (1H, 1H, 2H, 2H-perfluorooctyl)-silane). Although distinct hydrophobic/hydrophilic areas were obtained (as demonstrated by the sessile drop technique), these strategies were ineffective for the purpose of iMSC patterning (not shown).

Approach D used an inverse strategy. Based on a hydrophobic PS substrate (bacteriological grade), an oxygen plasma was applied for creating cell supportive areas. The remaining area was subsequently blocked by Pluronic, binding to hydrophobic areas only. This simple strategy provided stable, long-term patterning of iMSC#3 (>3 weeks) and allowed for adipogenic and osteogenic differentiation. The protocol is swift, operator-friendly, of low cost and does not produce harmful waste.

Table 5 Surface modifications tested for cell patterning

Strategy	**A**	**B**	**C**	**D**
Material	**glass**	**glass**	**glass**	**polystyrene**
Step 1	-	-	Silan deposition, liquid phase	-
Step 2	PDMS masking for selective treatment of different regions			
Step 3	Deposition of FN, Fluid phase	Silan deposition, vapour phase	Silan removal by plasma etching	Plasma oxidation
Step 4	Demasking			
Step 5	BSA blocking	Pluronic blocking		Pluronic blocking
Step 6	-	Conditioning with FN		-
Step 7	Cell seeding and attachment			
Patterning	**Ineffective**	**Ineffective**	**Initial efficiency**	**Stable patterning (>3 weeks)**
Cell condition	Cells alive	Cells alive	Cell death	Compatible with differentiation

Strategies using a glass substrate (A-C) were ineffective, while successful patterning was obtained using a PS substrate.

Box 9 Pluronics

Pluronics is a series of commercially available non-ionic surfactants. They are triblock copolymers, consisting of a central block of poly (propylene oxide) flanked by one block of poly (ethylene oxide) on each side, as described by the generic formula; (PEO)x-(PPO)y-(PEO)x. Given a hydrophobic surface immersed in an aqueous medium, the central PPO-block will adsorb spontaneously to the surface by hydrophobic interactions, while the hydrophilic tails (PEO-blocks) will extend into the medium and prevent protein adsorption and cell adhesion [200, 201].

Figure 14 Pluronic adsorption to hydrophobic surface
Drawing from Uppsala University

4.3 Proteins displayed by the BV173 pro B-cell line

Motivation & Strategy Our aim was to identify proteins produced by B- progenitor cells that may serve to mediate their communication with stromal cells and/or other microenvironmental factors. To narrow in the possible candidates, we aimed for detecting those proteins being displayed outside the cell, i.e. membrane and secretory proteins. Thus, we employed a method for cloning and expression of cDNA encoding such proteins based on their characteristic signal sequence (a hydrophobic, surface-directing "label"). We chose a variant based on retroviral expression (SST-REX) as this method was reported to be more efficient than previous methods.

Material We planned to subject primary B-cell progenitors to SST-REX screening, but we started off using a cell line for convenience (i.e. material access). The human cell line BV173 was selected to represent progenitor B-cells, based on evidence that these cells interact physically and functionally with stromal cells. When BV173 cells are grown in monoculture, they grow in suspension (free-floating) like any other hematopoietic cell line. In contrast, when co-cultivated with the stromal cells (MS-5 or BM MNC adherent cells), a fraction of the BV173 cells was found attached to the stromal layer, where they grew in the cobblestone-like pattern characteristic for stroma-interacting hematopoietic cells [202], **Figure 15**. Such an arrangement has been reported earlier [203].

Interestingly, we noted that the adherent BV173 cells had a slower proliferation rate than the non-adherent fraction (determined by CFSE tracking, unpublished). Moreover, phenotypic analyses by flow cytometry indicated that adherent cells were induced to proceed along their differentiation pathway. The BV173 cell line is at an early stage of B-cell differentiation [204], as demonstrated by expression of the pre BCR-component VpreB (**Table 6**, page 46) and CD34. Accordingly, neither monocultured BV173 nor the non-adherent BV173 cell fraction expressed IgM, belonging to the BCR. However, a fraction of the cells in the adherent population was IgM^+ and $CD37^+$, hence indicating maturation towards immature B-cells. However, an abnormal developmental progression was noted, as these cells co-expressed CD34. A hint of these results is mentioned in the discussion part of Article I, justifying the use of SST-REX on BV173 cells.

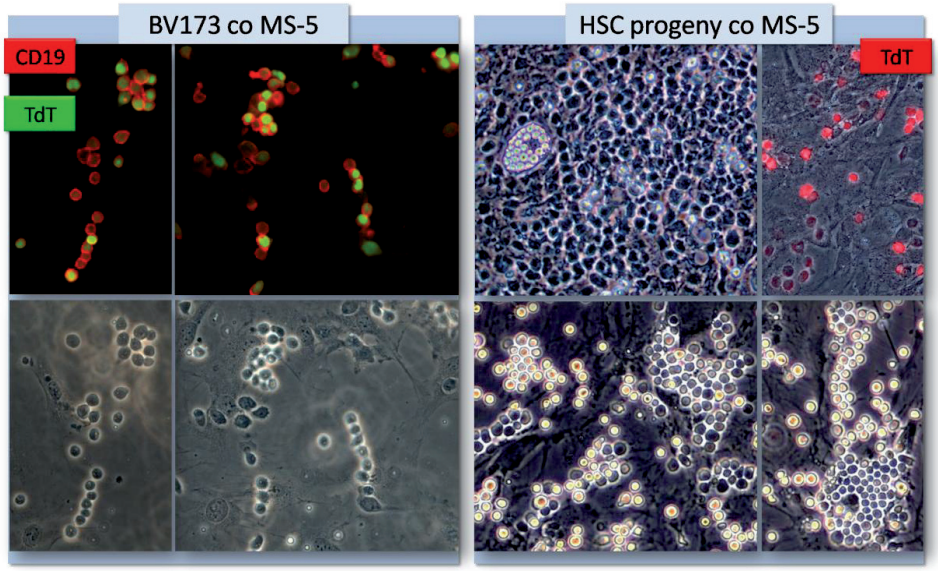


Figure 15 Hematopoietic-stromal interactions in culture Coloured pictures are fluroescent images, non-coloured pictures are phase contrast images. Upper right picture is a merge of the two types of imaging. Right panel: HSCs crawl beneath stromal cells in culture (pseudoemperipolesis) and form cobblestone areas of flat, phase-dark cells. This process is mediated by the stromal products CXCL12 (chemokine) and VCAM-1 (adhesion molecule), which cause attraction and binding of $CXCR4^+$/$VLA\text{-}4^+$ hematopoietic cells [202]. After cultivation for 2 weeks, some of the HSC progeny express TdT (red, upper right image). Cells on top of the stromal layer appears bright, as they remain spherical and thus refractile. Left panel: The pro B-cell line BV173 showed similar interaction with the stromal layer (MS-5 cells). Immunocytochemistry revealed heterogeneity in BV173 phenotype, as the cells ($CD19^+$, red plasma membranes) expressed variable levels of TdT (green, nuclei). *Unpublished images, Ellen Tenstad.*

The scope of this study would have matched with a further investigation of this cell line-based co-culture system, including an evaluation of its suitability as a convenient screening tool for identifying factors involved in the hematopoietic-stromal interaction. Factors exerting negative or positive influence on the observed scenario (adhesion and partial differentiation) would likely play a regulatory role in stroma-dependent B-lymphopoiesis.

Outcome Table 6 presents BV173 cDNAs that were identified by the SST-REX methodology.

Table 6 cDNAs expressed by B-cell progenitors (BV173 cell line) as identified by SST-REX methodology

No	cDNA encoding	Loc	#	NCBI ref
Known proteins				
1	VpreB	PM	1	NM_007128
2	HLA-DRb1	PM	2	V00522
3	HLA-DRb3	PM	3	U66825
4	Bone marrow stromal cell antigen 2, BST2 (CD317)	PM	4	NM_004335
5	Extracellular matrix metalloprotease inducer, EMMPRIN (CD147)	PM	1	NM_001728
6	Plasminogen activator urokinase receptor, PLAUR (CD87)	PM	1	XM_009232.1
7	Glucose transporter 10 (SLC2A10)	PM	1	NM_030777
8	Tumor rejection antigen 1 (gp 96)	PM/ER	1	NM_003299
9	Nucleobindin 1	Secreted	2	NM_006184
10	GM-CSF	Secreted	1	M11220
11	Ribophorin 1	ER	1	NM_002950
12	Heat shock 70kDa protein 5, HSPA5	ER	1	X87949
13	Oxygen related protein (ORP150)	ER	1	NM_006389
14	Cytochrome oxidase I	MT	1	NC001807
Novel proteins				
15	TMEM9	LE	1	NM_016456
16	Hu cDNA sequence from clone RP5-836N17	Unknown	1	AL049539
17	Uncharacterised protein KIAA0090	Unknown	1	XM_043712
18	BV105	Unknown	1	-

Loc: location, #: number of clones, Acc. No: NCBI Reference sequence, PM: plasma membrane, ER: endoplasmatic reticulum, MT: mitochondria, LE: late endosomes.

Characterisation of the TMEM9 protein showed localisation in intracellular membranes, thus excluding an obvious role in cell communication. However, intracellular proteins may also influence the extracellular environment. The closest homolog to TMEM9, named TMEM9B (also lysosomal), has been found to represent an essential module shared by inflammatory signalling pathways, i.e. the TNF, IL-1β and Toll-like receptor (TLR) pathways. Activation of these pathways results in the production of pro-inflammatory cytokines, and TMEM9B was found necessary for this process [205].

In 2003, TMEM9 cDNA was detected in a large-scale project called Secreted Protein Discovery Initiative. This project aimed for identifying novel secreted and transmembrane proteins for better understanding of intercellular communication, thus providing basis for new therapeutic strategies [206].

From the list of identified BV173 cDNA clones we suggest three candidate proteins for contributing to the hematopoietic-stromal interaction; CD317, CD147 and CD87. Of note, the two latter are both involved in ECM degradation, a process necessary for tissue reorganisation. Both factors recruit Matrix MetalloProteases (MMPs), which are enzymes that can digest extracellular proteins (extracellular matrix and cell adhesion molecules) and thereby release cell-cell contacts and ECM-bound growth factors. For instance, MMP-9 can unleash membrane-bound SCF and promote HSC differentiation and mobilisation [207]. It might be speculated that normal B-cell progenitors need these factors for survival and proper migration/allocation within the BM tissue.

Bone marrow stromal cell antigen 2 (BST2, CD317) This protein is expressed on multiple cell types, including stromal cells and developing B-cells. It is reported to be preferentially over-expressed in multiple myeloma cells [208]. Interestingly, fibroblasts induced to express BST2 showed enhanced supporting capacity towards a stroma-dependent murine pre B-cell line (DW34), an argument for a stimulatory role of BST2 in pre B-cell growth [209].

Extracellular matrix metalloprotease inducer (EMMPRIN, CD147) This glycoprotein belongs to the Ig superfamily. It is widely expressed and appears at especially high levels on human tumour cells. It is a pleiotropic molecule with roles in fetal development, T-cell activation and neurological processes. Originally, CD147 was identified as a tumour cell product that stimulated neighbouring fibroblasts to produce MMPs, thus possibly facilitating tumour invasion. CD147 has also been suggested to stimulate cell proliferation, endothelial growth factor (VEGF) production and tumour cell

glycolysis. An effect on multi-drug resistance, MDR is also reported. On this basis, CD147 is suggested as a potential therapeutic target in cancer [210, 211].

Plasminogen activator urokinase receptor (PLAUR, CD87) The receptor is a key molecule in the plasminogen activation system. This is an enzymatic cascade that results in ECM proteolysis. The process is initiated when CD87 binds urokinase (acronyms: PLAU, uPA), which in turn cleaves plasminogen, generating the active protease plasmin. Importantly, the cellular receptors involved restrict plasmin production to the immediate vicinity of the cell membrane. Additionally, CD87-integrin interactions can activate intracellular signalling, thus influencing cell adhesion, proliferation and differentiation [212, 213].

4.4 B-cell maturation niches in human BM.

Motivation This study was initiated thanks to interdepartmental exchange of knowledge and experience at DNR. Dr. Emina Torlakovic at the Department of Pathology challenged our literature-based understanding of HSCs being localised at the endosteal surface. According to her observations, no such relation was apparent in the human BM. In collaboration we wanted to investigate the distribution of B-cell progenitors within the BM and search for a relation to stromal elements.

Strategy & Methodology Stem cells/early progenitor cells are rare and difficult to discern in steady state conditions. In order to maximise the number of target cells, we selected reactive BM biopsies, originating from patients having increased hematopoietic demands due to environmental stress (e.g. infection or Hodgins disease). Importantly, the non-neoplastic nature of the BM samples was confirmed, excluding the possibility that our observations represented aberrant cell behaviour of malignant cells. Additionally, relevant findings were reproduced using BM biopsies from normal donors, ensuring their validity in normal hematopoiesis. Actually, malignant B-cell localisation was shown to deviate from the normal pattern.

Regular double-staining was performed, using two chromogen solutions. Additionally, we were able to perform triple immunoassays using the same two chromogens, due to a) use of markers with specific nuclear or cell surface localisation and b) mutually exclusive expression of markers that were stained by the same colour. E.g. brown colour was used to stain TdT and CD20; markers that are expressed in the nucleus and the plasma membrane respectively and never appear in the same cell (**Box 4**, page 15

and table and figure below). Technically, the antigens were labelled sequentially to exclude any possibility of nonspecific binding.

Table 7 Co-visualisation three antigens using two chromogens; brown and red, as indicated.

Staining	Nucleus	Plasma membrane	
"Triple 1"	TdT	CD20	Pax-5 (nucleus)
"Triple 2"	Pax5	VCAM-1	CD10 (Plasma membrane)

Outcome Pax-5 labelling of all developing B-lineage cells enabled us to demonstrate a peculiar spatial cell arrangement in marrows with numerous target cells. Accumulated BM B-cell progenitors were organised in strings. A similar finding had been reported in the mouse [214], but the authors did not indicate any relation to microenvironmental cells. Excitingly, we found that the B-cell strings coincided with the slender extensions of $CD10^+$ stromal cells. Furthermore, cell-stage specific markers showed a gradient in B-cell maturation towards the sinusoids. At steady state conditions, scarce B-cells also co-localised with $CD10^+$ cells, but a display of directed maturation was not possible due to low B-cell numbers. The results are interpreted as visualisation of stromal-dependent B-lymphopoiesis, **Figure 16**. This conclusion is strengthened by the finding that less stroma-dependent cells had no relation to $CD10^+$ stromal cells, i.e. other hematopoietic lineages, mature B-cells and malignant B-cells.

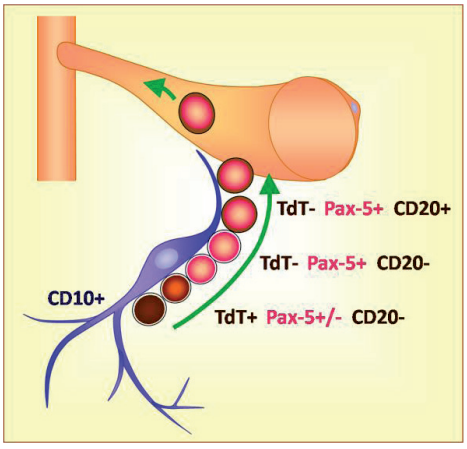


Figure 16
B-cell maturation in BM is supported by $CD10^+$ stromal cells and oriented towards the sinusoids. Conceptual drawing, summarising findings in Article II.

Cultivation studies have not given any clear consensus on what kind of cell(s) that support B-cell development in the body (see section 1.4.2, page 16). Our findings qualify slender $CD10^+$ cells as important niche cells for B-cell progenitors. Of note, CD10 expression has previously been detected on human fetal BM stromal cells [107]. Unexpectedly, we detected only partially expression of Vascular Cell Adhesion Molecule-1 (VCAM-1), which is reported to mediate B-cell support, **Table 2**, page 17. In contrast, the VCAM-1 marker defined another population of stromal cells, having a different morphology, no CD10 expression and no relation to B-lineage cells.

Perspective Further studies should characterise the slender $CD10^+$ stromal cells in more detail with respect to phenotype and expression of regulatory factors (**Table 2** and e.g. Wnt). Their spatial relation to HSCs should be evaluated (e.g. by CD133/CD10 co-staining), as HSC-supportive and B-supportive stromal cells seem related. Also, their relation to MSCs should be investigated (e.g. by CD146/CD10/Pax-5 co-staining). Even if stromal cells are commonly referred to as MSC descendants, the MSC population itself is an important candidate. Notably, MSCs are $CD10^+$ [39] and they are isolated from primary cultures similar to those used as feeder layers. In a comparative study, the fibroblast fraction (probably including MSCs, *own comment*) was found to sustain survival of immature B-cells as efficiently as the mixed population of adherent BM cells (i.e. fibroblasts, macrophages, endothelial cells and adipocytes) [126]. A recent review points out that the classical BM stromal cells have not been evaluated for differentiation capacity [215]. However, early observations (1989) lead to the conclusion that "*bone marrow stromal cells may thus represent at type of multipotent MSC, capable of further differentiation into adipocytes and possibly osteoblastic cells*" [216]. Recently, MSCs has successfully served as feeder layers for hematopoietic cells [217]. Our own indications promoting MSCs as B-cell supportive are: 1) The iMSC#3 cell line can serve as a feeder layer for cultivated B-cell progenitors [218] and 2) stromal cells lines (MS-5, OP9 and iMSC#3) differentiate spontaneously into adipocytes when left unpassaged.

Of important note, MSCs have been hypothesised to be identical to $CXCL12^+$ adventitial reticular (CAR) cells (see section 1.3.2, page 12). Precisely these CAR cells have been implicated as niche cells for the most immature B-cell progenitors in the murine system. In contrast, more mature stages (pro B and pre B-precursors) were in contact with another stromal population (IL-7-producing) [77, 219], possibly enforced by the stromal cell derived galectin-1 [220]. The final immature B-cell stage had no relation

to these stromal cells. Thus, stage-specific B-cell niches have been suggested in the murine BM.

In contrast, our observations on human BM indicate that a single stromal population is involved in support for all B-cell maturation stages, at least during increased hematopoietic demands. At such conditions, the linear arrangement allows for interactions between the developing B-cells. Homotypic interactions are less likely in steady state conditions, where B-cells appear individually (as estimated from the two-dimensional tissue section format). Although the results suggest that developing B-cells depend on docking to $CD10^{+}$ stromal cells, they may still receive mediators secreted from neighbouring cells of other phenotypes. Similar to what applies to HSC niches, it is likely that B-cell progenitors is influenced by various cells, both hematopoietic (e.g. macrophages) and non-hematopoietic. A recent cultivation study implements osteoblasts as a key component of the BM B-cell niche [114]. The finding that the BM anatomy allows stem/progenitor cells to receive simultaneous influences by endosteal and vascular/perivascular cells [79] may elucidate why no relation to bone was detected.

4.5 Effect of Wnt3a on human early B-lymphopoiesis

Motivation Contemporary murine studies suggested an important role of the canonical Wnt signalling in HSC renewal (see section 1.5.2, page 21) and also in B-lymphopoiesis [160]. Hence, we wanted to pursue this research in the human system. During the course of our investigation, this decision was promoted by microarray studies at our department, showing that normal and malign B-lineage cells of human origin had regulated expression of central Wnt pathway molecules (i.e. distinct expression levels at different maturation stages) [96].

Strategy & Methodology The first logical step was to determine whether BM B-lineage cells harboured the necessary "machinery" to elicit Wnt signalling, e.g. receptors and intracellular pathway molecules. Also, determination of Wnt sources would reveal whether the signalling was of autocrine or paracrine character. As few antibodies are available for detection of Wnt pathway molecules (due to evolutionary conservation), we were only able to detect and quantify expression at the mRNA level. The presence of mRNA is only indicative for protein expression, because of translational regulatory mechanisms. Hence, the most significant evidence of an operational pathway was provided by functional effects. The use of Wnt 3a as the triggering factor was again a

result of necessity, being the only factor commercially available at the time. Due to their limited solubility, Wnt proteins are difficult to produce and purify [137].

Outcome Transcripts encoding central Wnt pathway molecules were detected in primary B-cell progenitors and stromal cells (RT-PCR analyses). Further, the pathway was shown to operate in both cell populations. When subjected to Wnt3a in culture, the cells responded by stabilization of β-catenin, the key event necessary for transcription of Wnt-responsive genes, **Figure 6**, page 20. This was first demonstrated by Western blotting of lysates from primary BM B-cell progenitors (Article III). Later, immunocytochemistry confirmed that β-catenin accumulated in the nucleus of Wnt3a-responding cell lines, of both B-lineage and stromal origin, **Figure 17** and [167].

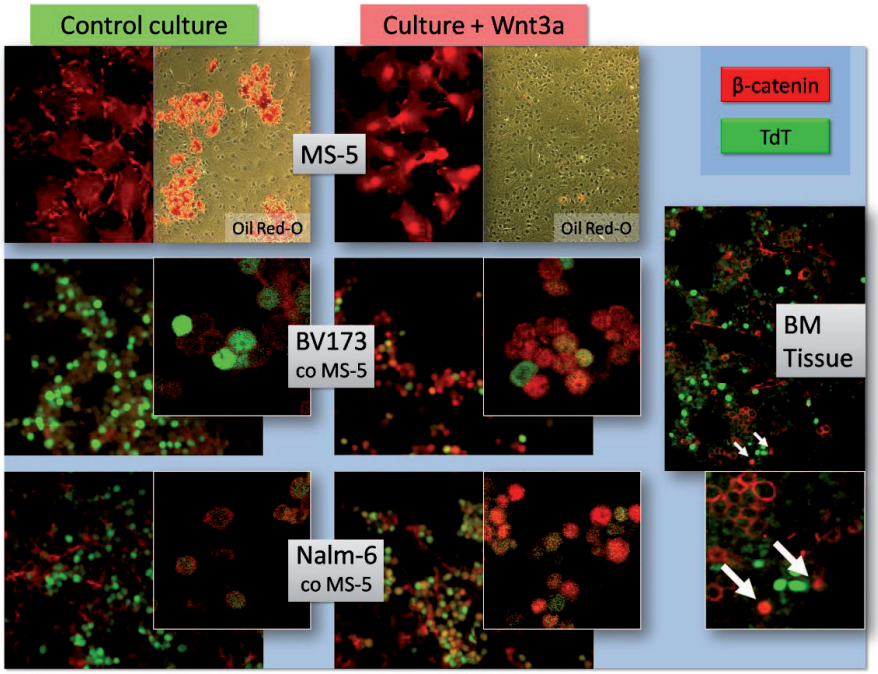


Figure 17 Nuclear β-catenin accumulation in cells exposed to Wnt3a in culture. At control conditions (left panel), β-catenin is localised to the cell membrane(red staining), where it plays a part in cell adhesion and is protected from degradation [221]. After Wnt3a exposure (100 ng/ml, 3h), β-catenin molecules accumulate in the nuclei (middle), as shown by red nuclear staining. TdT expression (green, nuclear) identifies leukemia cells, i.e. BV173 cells (representing pro B-cells) and Nalm-6 cells (representing pre B-cells, [222]). Yellow/orange staining appears when green and red staining co-localise. Wnt3a (100 ng/2 weeks) inhibited the spontaneous adipogenesis of MS-5 cells (upper right images, red staining identifies adipocytes). This effect was reversed by Dkk-1 (canonical inhibitor, not shown). BM tissue shows similar staining as control cultures, however two cells, likely of hematopoietic origin, display nuclear β-catenin (arrows, right image). *Unpublished images, Ellen Tenstad.*

Despite somewhat conflicting reports, canonical Wnt signalling is believed to help maintaining "stemness" of HSCs, restricting differentiation while allowing a degree of replication [164]. This view is compatible with our findings in Article III, where B-cell lymphopoiesis was repressed in the presence of recombinant Wnt3a. In fact, we observed that Wnt3a inhibited all hematopoietic differentiation (both myeloid and lymphoid) as well as adipogenic differentiation of stromal cells, see below. The inhibiting effect of Wnt on B-lymphopoiesis was substantiated by the following findings (unpublished):

- The effect was reproduced by another Wnt source
  (conditioned medium from L-cells transfected with Wnt3a).
- The effect was mimicked by LiCl
  (a Gsk3-inhibitor/β-catenin-stabiliser that serves as a canonical Wnt substitute).
- We observed a dose-dependent response
  (100 ng/ml Wnt3a reduced B-cell numbers more than a dose of 10 ng/ml).
- A similar trend was shown by analyses of the more immature TdT^+ progeny
  (cultures were harvested after 2 weeks instead of 3 weeks), and
- Similar results were obtained using a variant HSC/progenitor phenotype as the seeding population ($CD34^+$, $CD38^-$ BM cells).

A proliferative Wnt3a effect on the earliest progenitors was unlikely, as cobble-stone forming cells were typically scarce in treated cultures (unpublished).

Reversion of the Wnt3a effect by the use of inhibitors (sFRP/Dkk-1) confirmed its specific effect on B-cell progenitors (Article III). In contrast, these inhibitors were unable to rescue normal levels of B-cell differentiation in our B-lymphopoiesis assay (unpublished), opening up the possibility that Wnt3a may act upon HSC/progenitor cells via non-canonical pathway(s). Non-canonical signalling by Wnt3a has been reported previously [223]. It not unlikely that Wnt3a may act via several pathways and/or have distinct bioactivities on different developmental cell stages.

We did not test the sole effect of Wnt inhibitors in our cultures, an experiment that is worthwhile, knowing that both hematopoietic cells and stromal cells express Wnt protein transcripts. Such an experiment represents a "loss of function" strategy, similar to the one performed *in vivo* [158].

It has been suggested that Wnt may even reverse cellular differentiation [164], but this effect was not evident from our experiments. When added to B-cell progenitors in culture, Wnt3a did not affect maturation, which proceeded over time (day 0-14, as estimated by a reduction in CD34 expression), but had an inhibitory effect on cell proliferation (both pro B and pre B-cell stages), Article III.

Wnt signalling is functional in MSC and stromal cells, **Figure 17** and [224-226]. In our cultures, Wnt3a eradicated completely the spontaneous adipogenesis finding place in control wells (unpublished). Thus, the inhibitory Wnt-effect on B-lineage cells may have been a consequence of impaired support from Wnt-influenced stromal cells, as earlier suggested from murine studies [163]. As we were unable to maintain cultures without feeder layers, we could not determine whether Wnt acted directly or indirectly via stromal cells to reduce B-lymphopoiesis.

Perspective When submitted, our findings stood in sharp contrast to previous studies in the mouse performed by renowned laboratories. They were unexpected as Wnt3a was generally thought to lead to proliferation of stem/progenitor cells. Discrepancies may relate to different cell sources applied; we used primary progenitors from adult humans, while previous reports were based on more potent cells, i.e. murine fetal cells [160] and cells harvested from transgenic mice expressing the anti-apoptotic protein Bcl2 [137].

Due to the complexity of Wnt-related proteins and signalling pathways, we need much more information to decipher how they are involved in various processes. Lately, there has been a rising appreciation that Wnt signalling regulates hematopoietic cells in a context and dosage dependent way, as apparent from investigations of Wnt effects on HSC self-renewal. It is clear that the hematopoietic system is regulated by a complex set of regulatory molecules, which must be precisely balanced in order to keep homeostasis, **Figure 4**, page 12. Oversupply of single factors may cause unphysiological cell behaviours. As current *in vitro assays* are far from reflecting native microenvironments, there is a need for more advanced models. Specifically, acknowledgement of the morphogenic nature of important molecules (such as Wnts and Hedgehog) calls for strategies for obtaining controlled gradients in culture. Herein, microfluidic systems may come in useful (see next section).

Actually, we did some experiments on BM tissue cultures, containing fragments from BM aspirates. Here, B-cell progenitors resided in a more in-vivo like environment

and gradients could possibly form. CFSE-labelling and tracking were performed, aiming for detection of a shift in CFSE-distribution when comparing differentially treated samples. However, such analyses were disturbed by the experience that CFSE-labelling is not stably integrated, presumably due to catabolism of CFSE bound proteins [227].

4.6 Hematopoietic microenvironments on-chip

Motivation Several cell types and signalling molecules are believed to co-operate in BM niches where they are likely to form restricted spatial patterns (see section 1.3, page 7 and **Figure 4**). Use of microfabricated tools for localising cells and fluids may enable fabrication of stem cell niches and other tissue units with higher physiological relevance, thereby providing more realistic cell behaviour. Similar directions have been proposed in the literature [172, 228]. Microfluidic methods for exposing cells to gradients or isolated fluidic environments were initially used for studies on chemotaxis [180, 229] and neural axon growth [230, 231]. Lately, the use of gradients and other conditions enabled by microfabricated tools is being appreciated as promising strategies for emulating stem cell environments [232-235], like I envisioned when preparing my PhD project application in 2006. Potentially, a more in vivo-like environment may allow HSCs to maintain stemness, and progenitors to differentiate and multiply adequately. These properties have been difficult to conserve in current cultivation systems. Insufficient HSC numbers is a technical barrier that limits the use of stem cells for therapeutic transplantation [84].

Strategy We wanted to fabricate and evaluate a microfludic system that was able to sustain cells on a long-term basis, which is necessary for cell differentiation studies. Further, we wanted a system equipped with channels for creating heterogeneous laminar flow, thereby allowing for unique manipulation of the culture (see section 1.6, page 23). Thus, we implemented a published system meeting with these demands [236, 237]. Microcultivation using iMSC#3 cells was initiated, as both undifferentiated MSCs and their progeny serve to support stroma-dependent hematopoietic cells [27, 113, 114, 238]. Induction of adipogenic and osteogenic differentiation was performed for two purposes; 1) to test whether the system was compatible with normal cell behaviour, and 2) with the prospects of producing distinct hematopoietic microenvironments on-chip.

Outcome Two variant microfluidic systems based on PDMS microchannels bonded to a glass substrate was successfully fabricated. Experiments exploiting the channels for creating laminar flows demonstrated their capacity to provide for

heterogeneous conditions, both with respect to chemical environment and cellular composition, **Figure 7**, page 24.

However, these PDMS/glass systems did not allow for consistent differentiation of iMSC#3. We noted that the homogeneous substrate allowed cells to spread out in the chip, as they did not differ between areas designated for cultivation and those designed for perfusion. Cells astray are likely to cause a suboptimal environment over time by blocking the channels used for medium supply (these are kept narrow to allow slow perfusion). The problem was solved by making the perfusion areas non-supportive to cell growth, using selective surface activation and passivation. This modification involved the use of a PS substrate, as sustainable MSC#3 patterning was not obtained on glass (being the classical microfluidic substrate for PDMS systems), **Table 5**, page 43. The PDMS/PS systems maintained iMSC#3 healthy for more than 3 weeks - as demonstrated by a Live/Dead viability assay and normal differentiation processes. This outcome is superior to previous documentation of on-chip MSC differentiation, all using systems based on unpatterned glass substrates [239-242]. Thus, there is a need for re-evaluating the properties of microfluidic cultivation substrates.

In PDMS/PS microfluidic systems, normal MSC differentiation was obtained under constant flow conditions, even though perfusion is reported to affect MSCs [243]. Caution should be exercised regarding the use of laminar flows for localised cell treatment, due to potential shear stress effects on cells. In preliminary experiments, iMSC#3 was subjected to long-term and repeated exposures of laminar streams (i.e. overnight x 7), for the purpose of inducing adipogenic and osteogenic differentiation in separate regions. The cells survived, but differentiation was not achieved. However these results may relate to the use of the non-optimal PDMS/glass systems. Nevertheless, it may be that localised cell treatment by laminar streams is only suitable for processes that can be induced by pulse stimulations.

Immediate prospects The microfluidic system developed is ready for future manipulation of the cellular environment by heterogeneous laminar flow, taking the above precautions in use. A strategy for circumventing problems due to shear stress involves the integration [244] of a cell-supportive microporous membrane into the system, thus separating the cultivation chamber into two floors. In such an arrangement, cells can be grown on top of the membrane and at static conditions, while the underlying compartment can provide localised signals (obtained by heterogeneous flow and transmitted via the

membrane) [245]. Alternatively, other microchannel designs may be implemented for producing gradients that inflict minimal shear stress upon cells [246, 247].

A membrane-separated two-compartment system may also provide a strategy for determining whether stroma-supported hematopoietic cells are directly or indirectly affected by an agent. Stromal cells and hematopoietic cells can reside in different compartments while interacting via membrane pores [248, 249], for the purpose of exposing them to different chemical environments. In this case, flow must prevail, to keep diffusion from equalising the chemical difference between compartments.

A natural further development includes exploration of methods for obtaining 3D cell cultures, thus adding further authenticity to the system [250-253].

Interestingly, cell shape and cytoskeletal tension can modulate MSC differentiation, as shown when MSCs are forced to adapt certain patterns. When grown in a mix of adipogenic and osteogenic factors, adipogenesis was shown to occur in a) central areas of the pattern, and b) when the area was minimised, making the cell stay spherical. Correspondingly, osteogenesis was shown to occur at a) peripheral areas of the pattern, and b) when cell spreading was allowed [254, 255]. Notably, the presence of osteoblasts in outer areas and adipocytes in central areas resembles a section of a long bone. Using these principles, variant heterogeneous cultures of osteoblasts and adipocytes can be fabricated and tested for ability to support stroma-dependent hematopoietic cells. The swift cell patterning method developed in Article IV can facilitate the production of such cultures and keep the cells in place for a sufficient time period, which is an improvement as compared with a previous method applied to achieve MSC patterning [255].

Future prospects Conclusions drawn from models are only as valid as the models themselves. Microfabricated tools for making advanced hematopoietic beds may provide basis for better understanding of the basic processes regulating the niches, knowledge that is important in medicine and tissue engineering. By exploiting unique microscale features, one can eventually imagine a future microsystem that provides the characteristic spatial arrangement of the niche/niche borderline elements; produced by co-patterning of stromal cells of different phenotypes and differentiated treatment with soluble signals, including gradients of regulatory factors. If achieved in a way that sufficiently mimics the *in vivo* environment, the natural behaviour of stem cells may be reproduced; stem cell quiescence, self-renewal and differentiation, **Figure 18**. In the future, engineered niches may be used for stem cell amplification, drug-testing and toxicological screening.

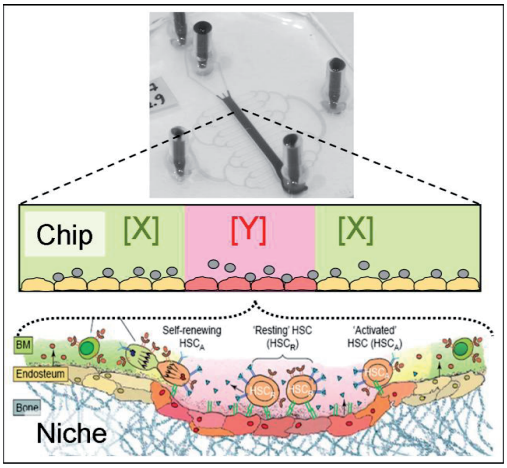


Figure 18 Use of niche technology to produce niches. Conceptual drawing of a HSC niche on a chip. Lower part is simplified from [256]. The focal streams may be exchanged with a gradient.

5 Conclusions

Proteins displayed by a human B-progenitor cell line (BV173) were successfully cloned by implementation of a signal sequence trap based on retroviral expression (SST-REX).

One unknown protein was characterised and named transmembrane protein 9 (TMEM-9). However, this protein was localised in intracellular membranes and has thus no obvious role in intercellular communication. We suggest three proteins as candidates for investigation of a possible role in stroma-dependent B-lymphopoiesis; bone marrow stromal cell antigen 2 (BST2, CD317), extracellular matrix metalloprotease inducer (EMMPRIN, CD147) and plasminogen activator urokinase receptor (PLAUR, CD87). EMMPRIN and PLAUR are both involved in degradation of ECM. Thus, they are able to facilitate cell migration and the release of immobilised growth factors.

The localisation of B-cell progenitors in human BM was determined by double and triple immunoassays. Visualisation of cell-stage levels showed a maturation gradient towards the sinusoids, but no relation to endosteal surfaces. Importantly, all benign B-cell progenitors harboured a niche formed by a population of slender $CD10^{+}$ stromal cells. Further investigation should characterise the phenotype of these stromal cells further and determine their contribution to stroma-dependent hematopoiesis. Diagnostic BM pathology may benefit from the ability to distinguishing between benign, stroma-attached B-cells and malign B-cells with no spatial stromal relation.

Human B-cell progenitors were shown to express Wnt pathway molecules on the transcriptional level, and activation of the canonical pathway was demonstrated by Wnt3a-induced accumulation of β-catenin. Functional cultivation assays showed that Wnt3a reduced both proliferation of B-cell progenitors and the production of B-cells from HSC. In our cultivation models, we conclude that Wnt3a acts as a negative regulator on B-lymphopoiesis, directly or via stroma. However, cells in their native hematopoietic microenvironment are likely to receive a delicate balanced combination of signals and may respond differently.

Microfluidic devices for cell cultivation were produced by soft lithography. MSCs were unable to differentiate properly in the original system, which was based on a homogeneous glass substrate. Hence, a PS-based system with a spatially defined cultivation area was developed. A well suited long-term environment for MSCs was obtained in the revised system, as shown by extensive adipogenic and osteogenic on-chip differentiation. The patterning strategy developed is more operation-friendly and efficient than previous methods. The device can be used for future stem cell research and enables manipulation of the cellular environment by heterogeneous laminar flow.

5.1 Concluding remarks

This study exemplifies how different methodologies complement each other when approaching a scientific problem. Manipulation of adult cells in culture allows for experimental flexibility and does not involve any ethical problems; however the results must be evaluated in terms of their context. Retroviral expression is a powerful tool for forcing eukaryotic cells to express foreign genes and may be further applied for genetic manipulation of hematopoietic cells, which is difficult to achieve by other methods. However great attention must be paid to safety issues. Immunohistochemistry permits investigation of cells in their native habitat and gives authentic and informative "snapshots". However, its applicability in human studies is restricted by limited opportunities for manipulating human beings. The use of microfabricated tools for obtaining completely new cell cultivation conditions is promising for performing "out-of-the-box" type experiments. Interdisciplinary communication will be crucial for making biologists appreciate the possibilities of this technology [257]. In general, future progress in research is likely to benefit from, and even depend on information flow across traditional barriers, thereby facilitating new concepts and technological solutions.

6 Supplements

6.1 Supplementary results

6.1.1 Supplementary information, Article IV

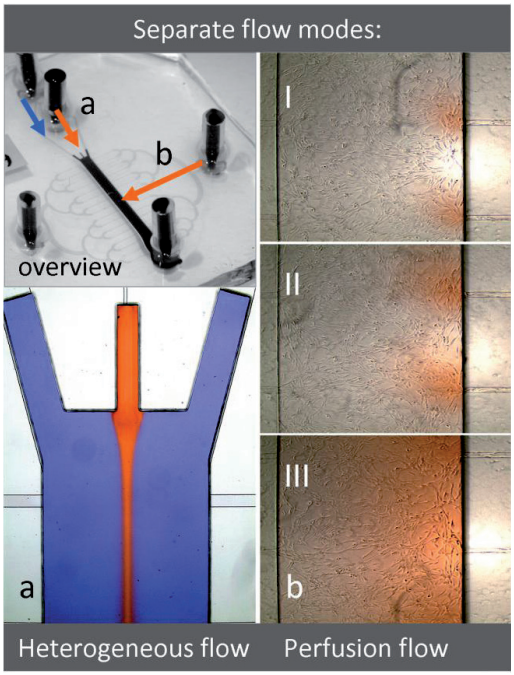


Figure S1 Alternative flow patterns By the use of dyes, two separate flow patterns in the microfluidic device are visualised. These are achieved by operating different sets of channels and are determining the composition of fluids in the cell cultivation chamber. Heterogeneous flows (a) for localised application of agents are obtained by feeding the upper inlets with different fluids and applying suction to the lower port, using a syringe pump. Perfusion flow (b, I-III are progressive images) for culture maintenance is performed by siphoning, using the distal ports (see text). Flow directions are indicated in the overview image (upper left).

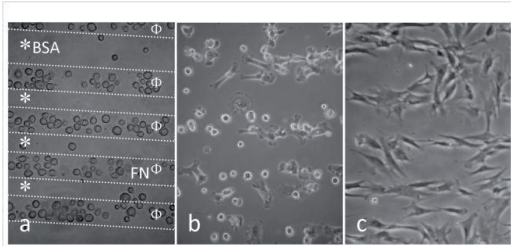


Figure S2 MSC localization is not restricted by BSA boundaries Progressive images of iMSC#3 cultivated in serum containing medium on a glass substrate that was coated alternately with FN (Φ) and BSA (∗) by the use of microchannels. Although cells initially adhered to FN coated regions only (a, 1h after seeding), they migrated out of these areas (b) and appeared unpatterned the next day (c).

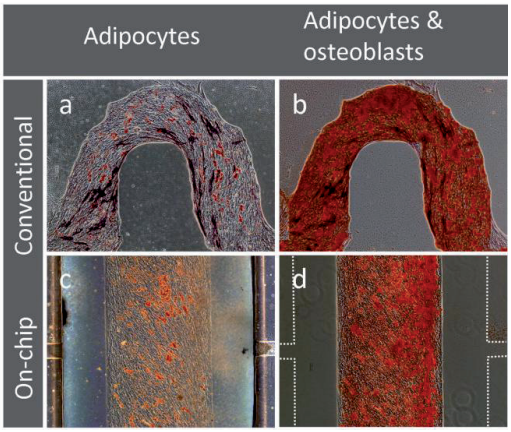


Figure S3 Simultaneous adipogenesis and osteogenesis on patterned PS substrates Dark field images showing one conventional (upper panel) and one microfluidic (lower panel) culture of iMSC#3 after 3 weeks in a 50:50 mixture of adipogenic and osteogenic medium. Adipocytes were stained by Oil Red-O (left panel) and subsequent staining of the same culture with Alizarin Red S revealed calcium produced by osteoblasts (right panel). The device top layer was removed for better end-point evaluation of the microfluidic culture, (d, dotted lines indicate former wall position) due to calcium deposition on the PDMS channel.

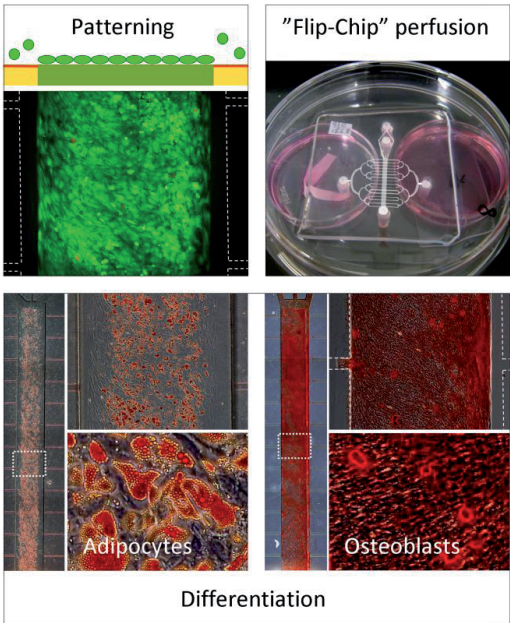


Figure S5 Conceptual figure in content pages (Lab Chip journal). Caption: We demonstrate the use of a microfluidic platform for long-term cultivation (3 weeks) of human mesenchymal stem-like cells (MSCs), a cell population of high interest for tissue engineering.

6.1.2 Perceptions of mold and microfluidic system in 3D

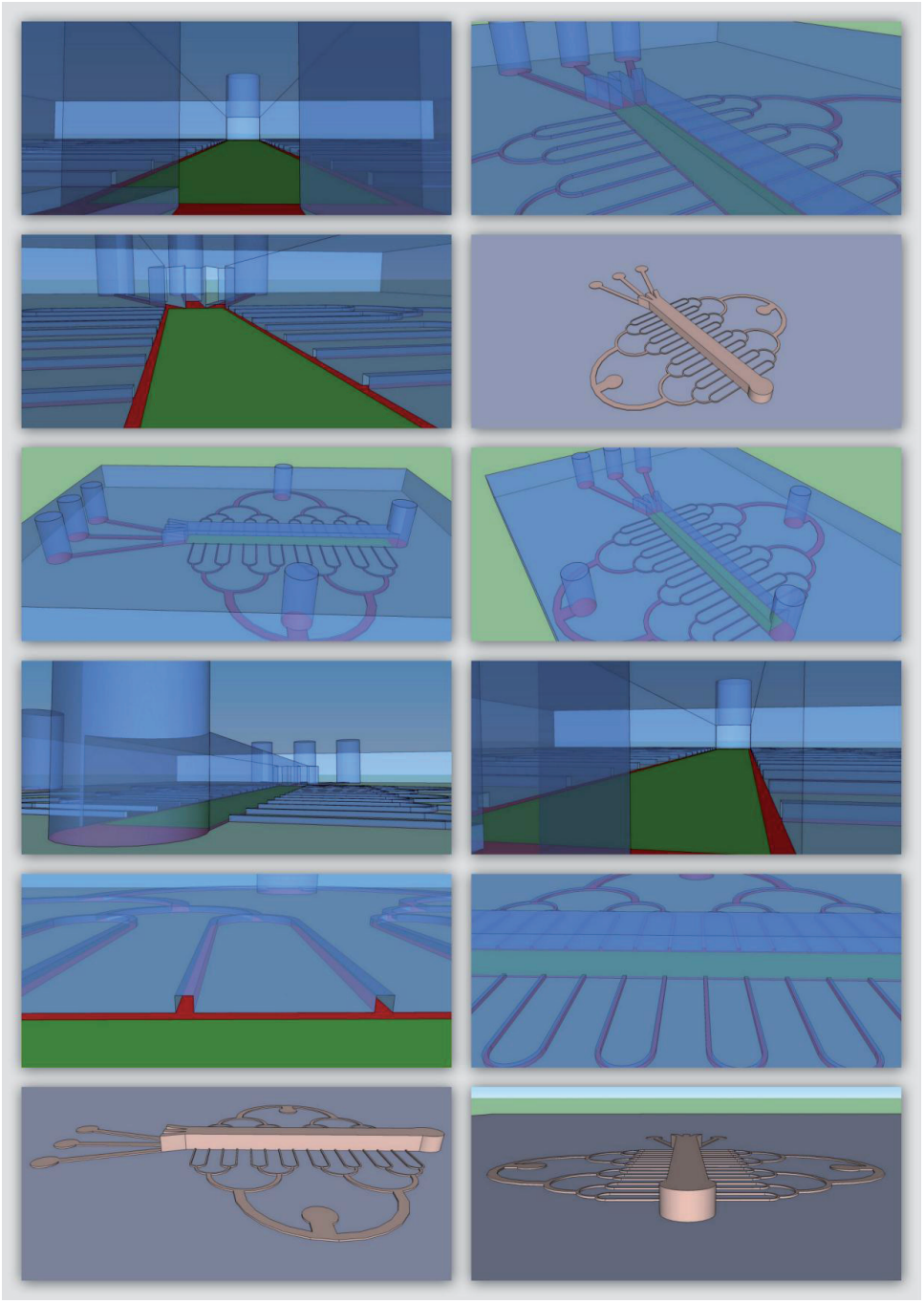


Figure S6 Green colour indicates cell-supportive areas and red colour indicates non-supportive areas. Drawings are not to exact scale. By Amund Tenstad (age 13).

6.1.3 Cell quantiation by flow cytometry

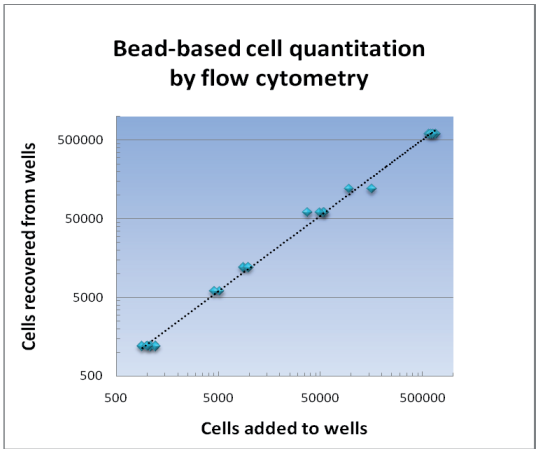


Figure S7 Proof of concept experiment for cell quantitation method. The numbers of aliquoted cells and rescued cells from each well are matching at all tested cell densities.

Protocol

Beads: Flow Cytometry Absolute Count Standard (Bangs laboratory Inc.)

Procedure & Estimation: A known number of beads is added to a cell sample*. A part of this sample is then analyzed by flow cytometry. The cells/beads fraction in the two sample parts remains identical (homogeneity is ensured by mixing before withdrawal and flow analyses).

$$\frac{\text{Cells recovered (unknown)}}{\text{Single-beads added}} = \frac{\text{Cell count by flow}}{\text{Single-bead count by flow}}$$

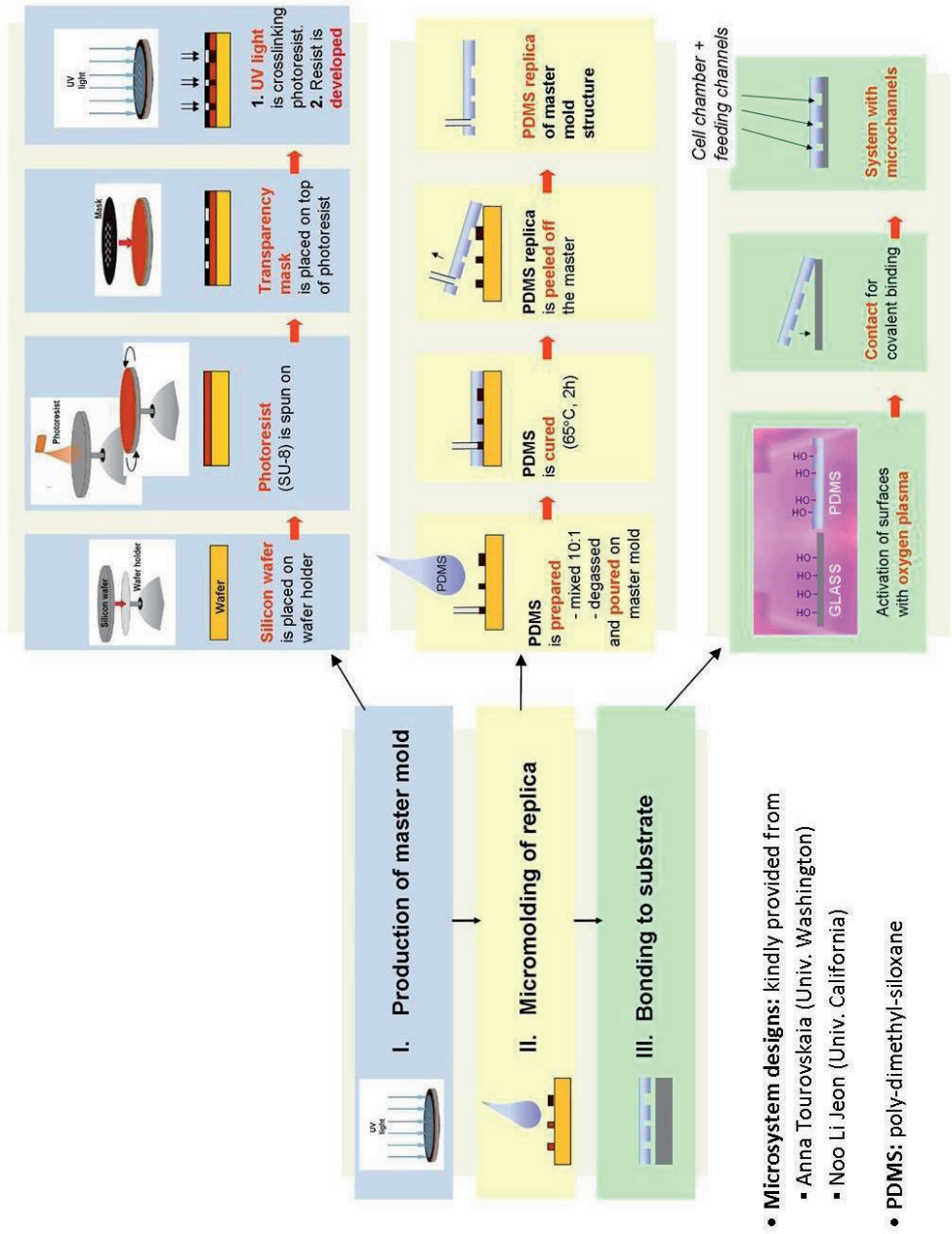
Some aggregation of beads occurred. The number of single beads added was determined by the following equation:

*Single-beads added =
% single beads (estimated by flow)/100 x [bead concentration**] x bead solution volume

** stated by the manufacturer

6.2 Soft lithography procedure

Schematic outline of photolithography, replica molding and bonding of PDMS to glass.



6.3 News piece on Article IV

RSC Publishing

Publishing

Highlights in Chemical Biology

Chemical biology news from across RSC Publishing.

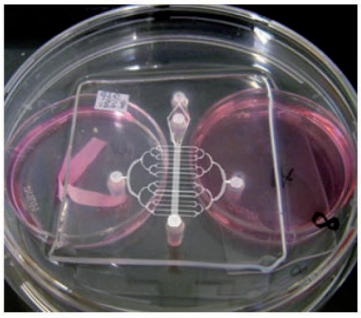
Stem cells find their polystyrene niche

29 March 2010

Norwegian scientists have developed a microfluidic platform to grow stem cells outside of the body in a controlled manner for a period of three weeks.

Stem cells from bone marrow are known as mesenchymal stem cells (MSCs) and like all adult stem cells they survive in specific microenvironments within the body, known as niches. But it's not so easy to grow them outside the body as they spread out as they grow which makes it difficult to control their microenvironment in vitro, as they tend to inhabit and block feeding channels. Previous microfluidic systems using glass substrates use hazardous chemicals to contain the cells, but Ellen Tenstad at the Vestfold University College in Horten and colleagues have used microtechnology to enable controlled experimental conditions that cannot be achieved using traditional large scale culture.

Tenstad modified polystyrene with selective oxygen plasma treatment and added a triblock copolymer to produce cell supportive and non-supportive areas. This allows the growth of the cells to be contained in the cell supportive area while the other areas could be used as feeding channels. The device can be made in a quick and user friendly way and allows the stem cells to be cultivated for three weeks, which is longer than previously reported. 'Such strategies may be used for making more representative in vitro models,' says Tenstad.



Stem cells find their polystyrene niche

Biju Parekkadan, an expert in stem cell biotechnology and immunology at the BioMEMS Resource Centre at Massachusetts General Hospital, Charlestown, US agrees that the device has potential, saying 'with further evaluation, these devices can enable basic biologists to study microenvironmental questions within the bone marrow stem cell niche at a realistic scale.'

Tenstad is enthusiastic about using microfluidics to achieve functionalities that are not currently possible, something that she has achieved with the help of several collaborators. Tenstad believes that 'MSCs have a great potential within research areas focusing on tissue engineering and immunomodulation.' Although Tenstad herself will be focussing on how these cells are involved in blood cell formation.

Laura Howes

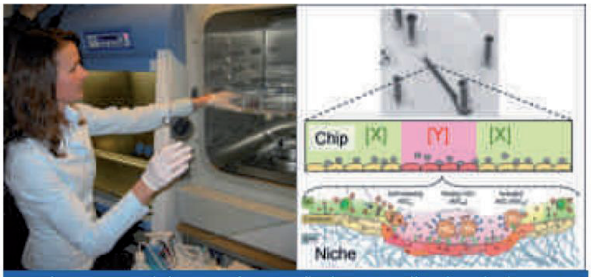
Enjoy this story? Spread the word using the 'tools' menu on the left or add a comment to the Chemistry World blog.

Link to journal article

Extensive adipogenic and osteogenic differentiation of patterned human mesenchymal stem cells in a microfluidic device
Ellen Tenstad, Ola Myklebost and Edith Rian, *Lab Chip*, 2010
DOI: 10.1039/b926738g

6.4 News piece on project

STEM CELLS ON-A-CHIP



At right: Conceptual drawing of a microfluidic stem cell niche. The lower part is simplified from Murphy et al. Trends in Cell Biology (2005)

Composed by the basic units of life, cell cultures are widely used in biomedical research, thereby avoiding the costs and moral dilemmas of animal experimentation. Insights in stem cell biology hold great promise for the future. Right management of stem cells may ultimately enable production of tissue units for therapy, and knowledge about cancer stem cells will hopefully bring more effective cancer treatments. However, traditional cell cultivation can only poorly reproduce the native microenvironments (niches) that are controlling stem cells, thereby limiting their potential.

At IMST, we have initiated an interdisciplinary project involving microfabrication for creating more representative models of stem cell niches in the bone marrow. Both the cellular composition and the chemical environment will be manipulated by exploiting the unique features of the microscale. For instance, by the use of laminar flow, multiple cell types can be precisely positioned within one culture and signals can be delivered locally (figure). Also, the microfluidic cell cultures can be exposed to gradients of stimulatory agents, (e.g cytokines and morphogens). These methods enable the controlled creation of heterogeneous on chip environments mimicking some of the complexity found in the body. Such models are likely to provide a better understanding of the fundamental processes regulating stem cells and hopefully contribute to progress within medicine. Future use of engineered niches may also include systems for drug-testing and toxicological screening.

Parts of our progress in this project has recently been disclosed in an Advance Article on the Lab on a Chip web site (DOI: 10.1039/B926738G), published by the Royal Society of Chemistry. The printed version is expected in May, with our artwork featured on the journal inside front cover. Moreover, the article will be reviewed in a news piece in "Highlights in Chemical Biology", which is another publication by the same publisher.

CONTACT
ELLEN TENSTAD : ET@HIVE.NO

2

In newsletter from Department of Micro and Nano Systems Technology (IMST), March 2010.

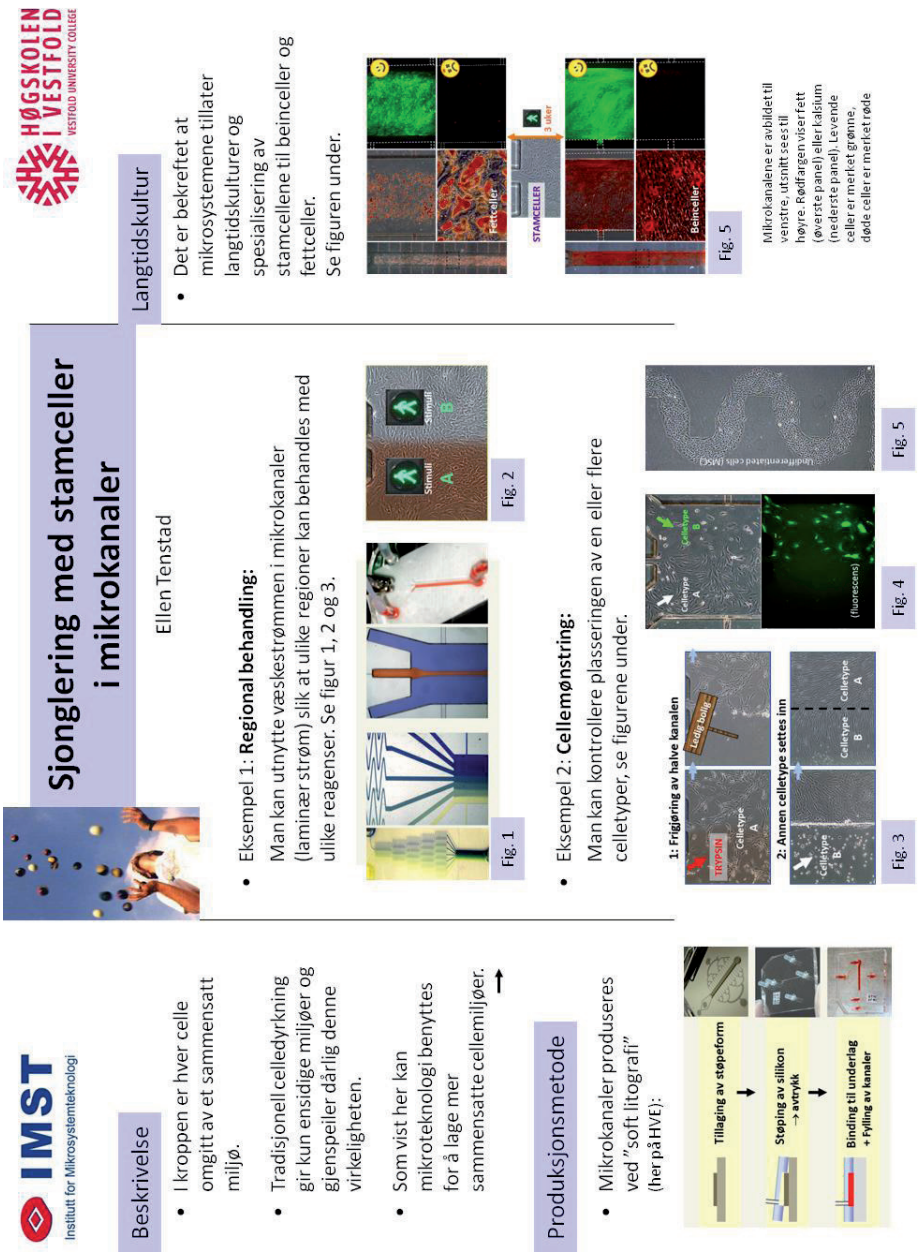
By Ellen Tenstad

6.5 Project Chart 2009

In ”Vestfold College Research summery, Institute for Microsystem technology”

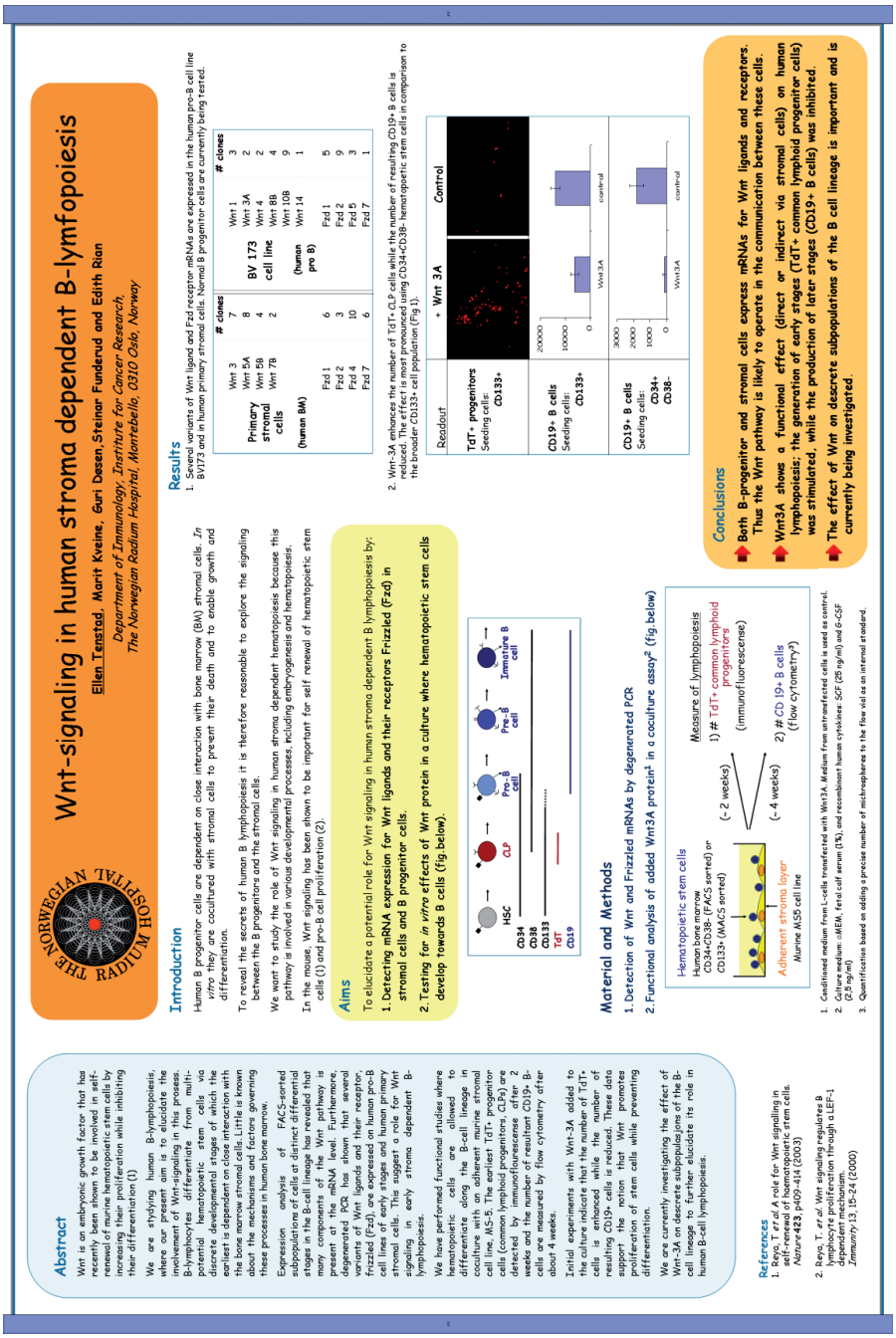
6.6 Popular Science Poster

Event: Norwegian Science Week, Høgskolen i Vestfold, 2009.



6.7 Scientific Poster

Conference: "Signal Transduction Determining the Fate of Stem Cells", Montana State University, Bozeman, Montana, 2003



6.8 For fun; a non-hematopoietic bed for developmental purposes

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Home »Home & Garden »Home Design & Decor »Home Decor »How to Make a Bed Properly

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How to Make a Bed Properly


Member

By Barbs2bits
User-Submitted Article
Article Rating: ★★★★☆ (8 Ratings)


Make a Bed Properly

There is a difference between merely flinging the linens over a bed and actually making it properly. Everyone should know how to make a bed, even if they don't care to do it all the time.

Instructions

Difficulty: Moderately Easy

Things You'll Need:

- bed
- fitted sheet
- flat sheet
- blanket
- bedspread or quilt
- pillows (http://www.ehow.com/shop_pillows.htm

1. Most linen sets come with a fitted sheet and a flat sheet. If you don't have a fitted sheet, follow the directions for the flat sheet and make a hospital corner in each corner. Fitted sheets are easy to put on flat if you put on diagonal corners at a time. Pick a corner, slip the corner of the sheet over it, make sure that the edge of the sheet is slightly under the mattress. Go to the opposite diagonal corner and fasten it. Move to another corner and then diagonal again. Make sure that the fitted corners are securely around the mattress and slightly under the edge of the mattress.


(http://i.ehow.co.uk/images/a04/fs/8t/bed-properly-1,2-800X800.jpg)

If the flat sheet has a 'right' and a 'wrong' side ie., decorative trim, finished edge of the hem, print on one side and not the other, etc. place the finished or right side down on the bed. Arrange the flat sheet so that it is flat and smooth on the bed with an equal amount hanging off each side and at least 12 inches of sheet hanging below the lower edge of the mattress.

Slightly lift the foot of the mattress and tuck the end of the sheet under the mattress, all the way across, make sure it is laying flat under the mattress. Take the corner of the sheet, which is hanging down toward the floor, grasp the corner and pull it taut away from the bed and then lay it over the top edge of the side of the bed, forming a triangle. Hold onto this tip of the triangle while you tuck the side of the sheet under the side of the mattress, from the corner of the mattress up the side a couple of feet. Then pulling the corner that you are holding taut, pull it down toward the floor at the corner of the bed and tuck it tightly under the mattress. This will form a hospital corner or fold right at the corner of the mattress and secure the flat sheet. Repeat on the other side.


(http://i.ehow.co.uk/images/a04/fs/8t/bed-properly-1,3-800X800.jpg)

Fluff the blanket over the bed and secure it with hospital corners exactly like you did the flat sheet. Smooth it out flat. Fold back the upper end of the blanket (at the head of the bed) so that it is folded toward the foot of the bed and the edge of the fold is approximately at the edge of the pillows. Pull the flat sheet taut under the blanket and fold the upper edge of the flat sheet down over the blanket, following the fold line of the blanket, so they are folded together. Now the right side of the flat sheet will show where it lays over the blanket.

4. Pillow slips go on the pillows which are arranged at the head of the bed, flat and with the open ends of the pillow cases toward the outer edges of the bed. This displays any decoration on the edge of the pillow case.

5. The bedspread or quilt goes on next, arranged to hang over three sides of the bed evenly and just touch the headboard. Tuck the bedspread slightly under the edges of the pillow, making sort of a pleat along that edge.


(http://i.ehow.co.uk/images/a04/fs/8t/bed-properly-1,6-800X800.jpg)

Decorative pillows can be arrange on top of the pillows and however you like them.

Tips & Warnings

- Always stretch sheets slightly taut when you are putting them on or arranging the corners, this will keep them flat and unwrinkled.

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Articles I-IV

I

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III

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Wnt expression and canonical Wnt signaling in human bone marrow B lymphopoiesis

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Abstract

Background: The early B lymphopoiesis in mammals is regulated through close interactions with stromal cells and components of the intracellular matrix in the bone marrow (BM) microenvironment. Although B lymphopoiesis has been studied for decades, the factors that are implicated in this process, both autocrine and paracrine, are inadequately explored. Wnt signaling is known to be involved in embryonic development and growth regulation of tissues and cancer. Wnt molecules are produced in the BM, and we here ask whether canonical Wnt signaling has a role in regulating human BM B lymphopoiesis.

Results: Examination of the mRNA expression pattern of Wnt ligands, Fzd receptors and Wnt antagonists revealed that BM B progenitor cells and stromal cells express a set of ligands and receptors available for induction of Wnt signaling as well as antagonists for fine tuning of this signaling. Furthermore, different B progenitor maturation stages showed differential expression of Wnt receptors and co-receptors, β-catenin, plakoglobin, LEF-1 and TCF-4 mRNAs, suggesting canonical Wnt signaling as a regulator of early B lymphopoiesis. Exogenous Wnt3A induced stabilization and nuclear accumulation of β-catenin in primary lineage restricted B progenitor cells. Also, Wnt3A inhibited B lymphopoiesis of $CD133^{+}CD10^{-}$ hematopoietic progenitor cells and $CD10^{+}$ B progenitor cells in coculture assays using a supportive layer of stromal cells. This effect was blocked by the Wnt antagonists sFRP1 or Dkk1. Examination of early events in the coculture showed that Wnt3A inhibits cell division of B progenitor cells.

Conclusion: These results indicate that canonical Wnt signaling is involved in human BM B lymphopoiesis where it acts as a negative regulator of cell proliferation in a direct or stroma dependent manner.

Background

In mammals, the early antigen independent phase of B lymphopoiesis takes place in the intersinusoidal spaces in the bone marrow (BM). Here, the B cell progeny mature from hematopoietic stem cells (HSC) via early lymphoid progenitors (ELP, comprising common lymphoid progen-

itors and early B), pro-B, pre-B and immature B developmental stages characterized by successive steps in the rearrangement of immunoglobulin genes and consecutive expression of cellular markers [1-3]. Using immunohistochemical doublestaining we have revealed earlier that all developmental stages of the B cell lineage in human BM tissue are in close contact with slender $CD10^+$ stromal cells or their extensions [4]. This finding correlates with the consensus that B lymphopoiesis is tightly regulated by signals provided by mesenchymal stromal cells and components of the intracellular matrix in the BM microenvironment *in vivo* [4-6]. However, the elements of this signaling are yet inadequately identified; stromal factors like IL 7, Flt3 ligand [7], IL-3 [8,9] and SDF1 [10,11] are essential, but not sufficient for BM B lymphopoiesis [2]. Clearly, there is a need for further characterization of both the stromal phenotype as well as the autocrine and paracrine factors that participate in the regulation of BM B lympopoiesis.

Wnt proteins belong to a large and highly conserved family of secreted, cystein-rich glycoprotein signaling molecules, consisting of 19 members. They are likely to act locally because of their limited solubility [12] and tendency to associate with the cell surface extracellular matrix [13]. Signaling is initiated by Wnt proteins binding to receptors of the Frizzled family (Fzd) on the cell surface. This binding is promiscuous and the ligand/receptor specificities are not yet properly determined. Depending on particular Wnt/Fzd combinations, at least three signaling cascades may be activated. Most studied is the canonical Wnt pathway, which is activated by members of the Wnt1 class (such as Wnt1, Wnt2, Wnt3 and Wnt8) [14]. A key regulatory molecule in this pathway is β-catenin, which in the absence of a Wnt signal is kept low through continuous phosporylation by glycogen synthase kinase-3β (GSK-3β), resulting in a subsequent proteasome dependent destruction of β-catenin. Binding of Wnt ligands to Fzd receptors and coreceptors LRP5/6, leads to inactivation of GSK3β and thereby accumulation of nonphosphorylated β-catenin, which enter the nucleus. Here, β-catenin acts as a coactivator of members of the lymphoid enhancer factor-1 (LEF-1)/T-cell factor (TCF) family of transcription factors to stimulate transcription of Wnt target genes [15]. Activation of Wnt signaling can be inhibited by soluble antagonists, including the Dickkopf (Dkk) family and the soluble Fzd related proteins (sFRP) [16].

Recently, Wnt proteins have drawn attention as a set of factors operating in embryonic development, growth regulation of adult tissues and cancer formation [15,17-20]. Moreover, Wnt signaling plays a central role in the communication between HSC and stromal cells [21] as well as in several other stem cell niches [22,23]. Several observations have established direct roles for Wnt signaling in the maturation process where hematopoietic stem cells lose their pluripotency and commit to specific lineages [24-26]. LEF-1 and Fzd9 knockout mice show defect B lymphopoiesis [24,27] and Wnt signaling seems to be involved in development of leukemia [28-30] and malignant myeloma [31]. Moreover, in murine B lymphopoiesis this signaling pathway has a stimulatory effect on pro-B cells from fetal liver [24]. As early B lymphopoiesis in mice and humans to a certain extent shows distinct factor dependency [32], and since fetal and adult lymphopoiesis takes place in different maturation niches, the aim of the present study was to investigate Wnt signaling in human BM B lymphopoiesis in more detail. We have examined which Wnt signaling pathway molecules that are expressed in B progenitor cells and stromal cells from human BM, and analyzed the regulated expression of several Wnt receptors (Fzd and LRP), β-catenin and plakoglobin as well as the central transcription factors LEF-1 and TCF-4 during the early B lymphopoiesis. Furthermore, we have investigated the effect of recombinant Wnt3A on progenitor B cells. We found that Wnt3A induced β-catenin stabilization and inhibited *in vitro* B lymphopoiesis in a coculture with stromal cells by suppression of initial cell proliferation. Thus, canonical Wnt signaling may be involved in human BM B lymphopoiesis.

Results

A distinct set of Wnt ligands, Fzd receptors and Wnt antagonists is expressed in B progenitor cells and stromal cells from human BM

Previous work has demonstrated expression of Wnt5A, Wnt2B and Wnt10B in pooled human BM populations [26]. However, the expression pattern of Wnt ligands, Fzd receptors and Wnt antagonists in human B lineage cells has not been explored. In the absence of available antibodies to detect these large families of proteins, we performed conventional RT-PCR on RNA isolated from FACS sorted B progenitor cells ($CD10^+IgM^-CD45^+$) pooled from three different donors, using primers designed specifically to detect mRNA expression of all known Wnt ligands and Fzd receptors as well as the Wnt antagonists Dkk1, Dkk4, sFRP1-4 and WIF1 (fig. 1 and table 1). In B progenitor cells, Wnt 2B, 5B, 8A, 10A and 16 mRNAs were readily detected. Interestingly, the Wnt16 PCR product had two bands of 520 bp and 233 bp, respectively (fig. 1). The 520 bp band represents the full-length form and the 233 bp band represents a possible splice variant lacking exon 3, potentially giving rise to a truncated Wnt16 form. In addition, expression of several other Wnt mRNAs was detectable, however, less readily (table 1). The Fzd receptors showed on average much higher mRNA expression levels than the Wnts, where Fzd2, 3, 4, 5, 6 and 9 mRNAs were easily detectable in the B progenitor population, as demonstrated by strong PCR bands. Fzd1 and Fzd7 mRNA

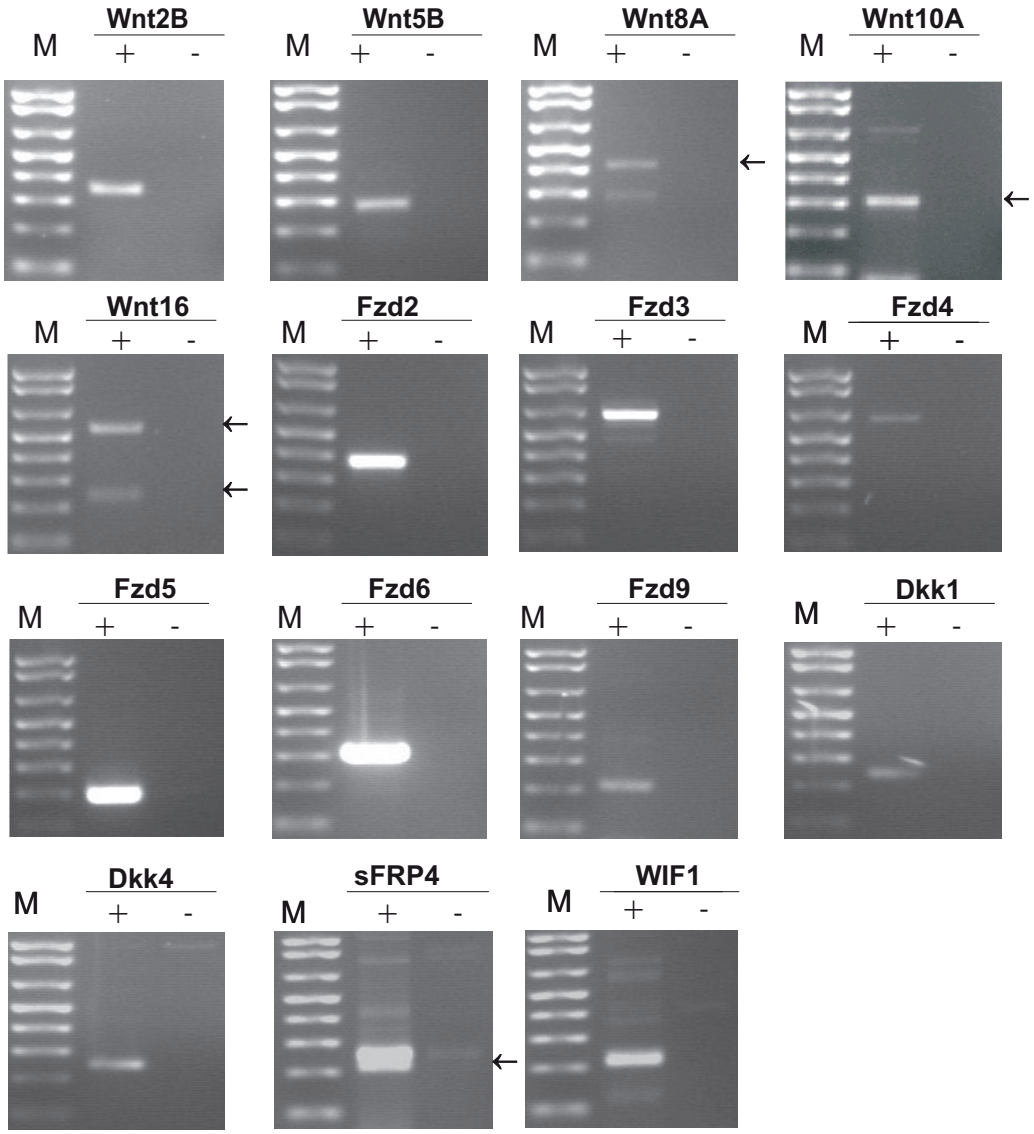

Figure 1

mRNA expression analyses of Wnt ligands, Fzd receptors and Wnt antagonists. RT-PCR detection of mRNAs for Wnt ligands, Fzd receptors and Wnt antagonists in BM B progenitor cells. The + and - symbols indicate the presence and absence of reverse transcriptase in the reaction mix, respectively. One representative of two experiments is shown. Amplicon sizes: Wnt2B: 328 bp, Wnt5B:279 bp, Wnt8A: 400 bp, Wnt10A: 296 bp, Wnt16: 520 bp, Fzd2: 306 bp, Fzd3: 622 bp, Fzd4: 605 bp, Fzd5: 197 bp, Fzd6: 300 bp, Fzd9: 210 bp, sFRP4: 243 bp, WIF1: 200 bp, Dkk1: 235 bp, Dkk4: 241 bp. M: Size marker 1 kb Plus DNA ladder (Invitrogen, USA). Where two different bands are detected, an arrow marks the correct band.

Table 1: mRNA expression of Wnt ligands 1–19, Fzd receptors 1–10, Wnt antagonists sFRP1-4, WIF1, Dkk1 and Dkk4

	BM B progenitor cells	BM stromal cells (BMS)	Human fetal brain		BM B progenitor cells	BM stromal cells (BMS)	Human fetal brain
Wnt1	+/-	-	+	Fzd1	+/-	-	-
Wnt2	-	-	+	Fzd2	+	-	+
Wnt2B	+	+	+	Fzd3	+	+	+
Wnt3	-	-	+	Fzd4	+	+	+
Wnt3A	+/-	-	-	Fzd5	+	-	+
Wnt4	+/-	-	+	Fzd6	+	+	+
Wnt5A	+/-	+	+	Fzd7	+/-	-	+
Wnt5B	+	+	+	Fzd8	ND	-	ND
Wnt6	ND	-	ND	Fzd9	+	-	+
Wnt7A	-	-	+	Fzd10	-	-	-
Wnt7B	-	-	+	Dkk1	+	+	+
Wnt8A	+	-	+	Dkk4	+	-	-
Wnt8B	ND	+	ND	sFRP1	-	-	+
Wnt9A	+/-	-	+	sFRP2	+/-	+	+
Wnt9B	+/-	+	+	sFRP3	+/-	+	+
Wnt10A	+	-	-	sFRP4	+	-	+
Wnt10B	+/-	-	+	WIF1	+	ND	+
Wnt11	+/-	-	+				
Wnt16	+	-	+				

Genes expressed (+), not expressed (-), variably expressed between experiments(+/-), not determined (ND). N = 2. BM B progenitor cells: $CD10^+IgM^-CD45^+$ cells sorted by FACS and pooled from three different donors. Total RNA from human fetal brain was used as control.

expression was also demonstrated, but at lower levels than the other Fzds (table 1). We also detected expression of the Wnt antagonists Dkk1, Dkk4, sFRP4 and WIF1 mRNAs in the BM B progenitor cells (fig. 1 and table 1). Of these, sFRP4 mRNA was most readily detectable, suggesting the highest expression level. sFRP2 and sFRP3 mRNAs were variably detected (table 1), suggesting low expression levels.

RT-PCR performed on RNA from BM stromal cells showed expression of Wnt2B, Wnt5A, Wnt5B and Wnt8B. mRNA expression of Wnt9B was also demonstrated in these cells, although at a lower levels. Moreover, Fzd3, 4 and 6 mRNAs were detected in BM stromal cells, as well as expression of the Wnt antagonists Dkk1, sFRP2 and sFRP3 mRNAs (table 1).

Regulated expression of Wnt receptors, β-catenin, plakoglobin, LEF-1 and TCF-4 mRNAs during human BM B lymphopoiesis

Identification of differential expression of Wnt signaling molecules during the B lymphopoiesis may reveal at which window in the process Wnt signaling is active. Thus, using quantitative real-time PCR, we examined the expression of a selection of Wnt receptors, β-catenin, plakoglobin and transcription factors in FACS sorted human BM B lineage cells representing different maturation levels; ELP cells ($CD10^+CD34^+CD19^-$, also tested to be $CD38^+$), pro-B cells ($CD10^+CD34^+CD19^+CD20^-IgM^-$), large pre-B cells ($CD10^+CD34^-CD19^+CD20^{dim}IgM^-$), small pre-B ($CD10^+CD34^-CD19^+CD20^-IgM^-$) and immature B cells ($CD10^+CD34^-CD19^+CD20^+IgM^+$). Due to limited number of cells, expression analysis in ELP cells was restricted to seven out of ten mRNAs.

The results showed regulation of several of the important Wnt-signaling molecules, and different expression profiles were recognizable (fig. 2). mRNA levels for the plasma membrane receptors LRP5, LRP6, Fzd5 and Fzd6 dropped considerably as the cells develop from small pre-B cells into immature B cells. Furthermore, Fzd5 mRNA levels were strongly up-regulated as the cells commit to the B lineage (from ELP to pro-B), with a further up-regulation as the cells differentiate to pre-B cells. Fzd2 and Fzd9 mRNA levels, on the other hand, seemed to increase somewhat throughout the differentiation, with highest levels in small pre-B and immature B cells. In small pre-B cells, the mRNA levels of LRP5 and Fzd9 were about two-fold higher than in the large cycling pre-B cells. The expression levels of all receptors were low compared to the expression levels of e.g. LEF-1 and β-catenin, indicating relative low mRNA expression levels. Fzd3 and Fzd4 mRNAs were not detectable with the amount of RNA template used in these assays.

The mRNA expression of β-catenin and plakoglobin showed little variation as the cells differentiate. β-catenin mRNA was evenly expressed in ELP, pro-B, large pre-B and immature B, with a small increase (near two-fold) in small pre-B cells. Plakoglobin mRNA levels, in contrast,

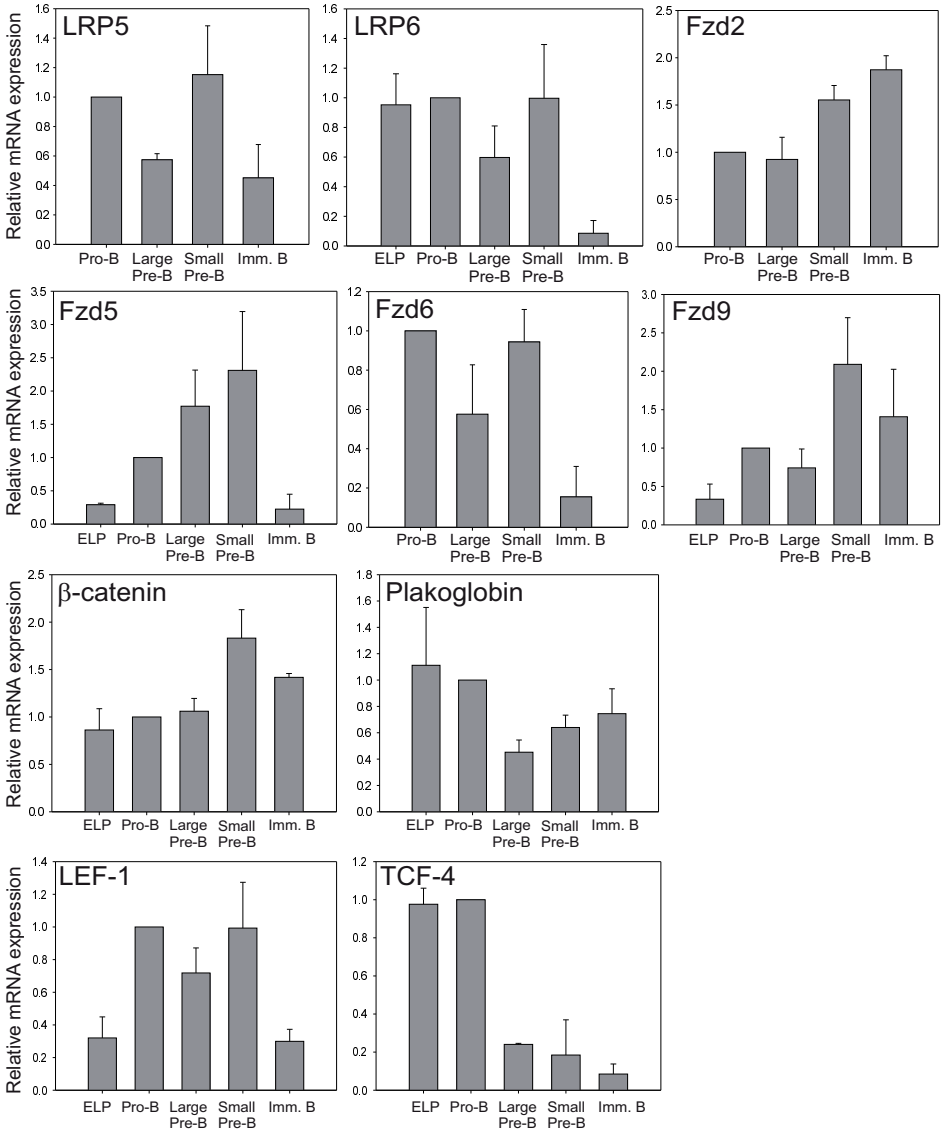

Figure 2

Real-time PCR analysis of relative mRNA expression levels of Wnt pathway molecules in BM B progenitor sub-populations. The sub-populations ELP, pro-B, large pre-B, small pre-B and immature B (imm.B) were isolated by FACS sorting. The relative mRNA expression levels of Wnt receptors and co-receptors, β-catenin, plakoglobin, LEF-1 and TCF-4 were quantified by real-time PCR analysis. Calculations of the expression levels were performed using the standard curve method and then normalized to the expression of PGK1 mRNA. mRNA levels in pro-B cells were used as calibrators. The bars represent the mean of 3–5 experiments ± SEM.

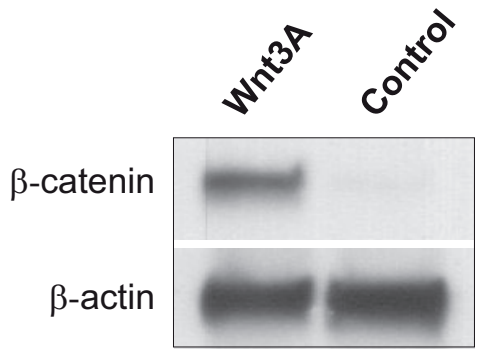

Figure 3
Wnt3A induces β-catenin stabilization in BM B progenitor cells. Western blot analysis of β-catenin levels in BM CD10$^+$ B lineage progenitor cells stimulated with Wnt3A (100 ng/ml) or vehicle (PBS with 0.1% detoxified BSA) for 3 hours. The blots were incubated with an Ab against β-catenin, followed by an Ab against β-actin to ascertain equal loading in the wells. The same results were found in cells from 4 out of 5 different donors, indicating some degree of donor variation in the response to Wnt3A.

decreased 2-fold as the cells became large pre-B cells (fig. 2).

LEF-1 and TCF-4 mRNA expression is highly regulated during the early B lymphopoiesis, as shown previously by microarray analysis (Hystad ME *et al*, manuscript in preparation and [33]). Our results showed a strong up-regulation of LEF-1 mRNA as the cells commit to the B lineage, and the expression was kept continuously high until the cells become immature B cells, where the level was reduced to the same as in uncommitted progenitors. Here, low LEF-1 expression was further confirmed by the absence of LEF-1 protein in B lymphocytes from peripheral blood (results not shown). The relative TCF-4 mRNA levels, on the other hand, were high in both ELP and pro-B, and decreased (up to 5-fold) as the cells passed through Ig rearrangement (pre-B – immature B cells) (fig. 2). It should be noted that the LEF-1 mRNA expression was detected 5–8 cycles earlier than the TCF-4 mRNA expression, indicating that LEF-1 mRNA is much more abundant than TCF-4 mRNA.

Wnt3A induces β-catenin stabilization and accumulation in BM B progenitor cells

Our data demonstrated that human BM B progenitor cells express a set of central players in the canonical Wnt signaling pathway, potentially allowing a Wnt signal to be conveyed. To further examine whether B progenitor cells could respond to treatment with Wnt proteins, we looked for the stabilization and subsequent accumulation of the vital signaling molecule β-catenin in CD10$^+$ B progenitor cells. When these cells were treated with Wnt3A, the amount of β-catenin increased substantially compared to the very low levels in untreated cells (fig. 3). Although there were some donor variations, the results showed that the B progenitor cells are able to receive and communicate a signal from the Wnt pathway.

Wnt3A inhibits human in vitro *B lymphopoiesis*

Having identified expression of central molecules in the canonical Wnt pathway in BM B progenitor cells, we performed two variants of B lymphopoiesis assays to investigate whether Wnt signaling (using recombinant Wnt3A) had a functional effect on B lymphopoiesis *in vitro*. Both assays were based on coculture with the murine stromal cell line MS-5. In assay 1 hematopoietic progenitor cells (HPC) were tested for their capacity to develop into B lineage cells, whereas in assay 2 B progenitor cells were measured for survival and expansion. At the endpoint of the assays, each sample was subjected to quantitative flow cytometry and the total number of cells positive for the pan B cell marker CD19 was measured. In assay 2, analysis of the differentiation marker CD34 was included.

Initial analyses demonstrated that Wnt3A had an inhibitory effect when BM HPC (CD133$^+$CD10$^-$) were grown on stromal cells for 3 weeks at conditions that favored B lymphopoiesis (assay 1). The number of CD19$^+$ cells in the samples treated with Wnt3A was 5 times less than the number measured in the control samples (fig. 4A). The inhibited B lymphopoiesis could result from Wnt3A suppressing differentiation of the HSC pool found in the HPC population [34], an indirect effect mediated by the stromal cells [35], or, alternatively, Wnt3A could target more committed lymphoid progenitor cells. To examine the latter possibility in more detail, we tested whether Wnt3A acted on later stages of *in vitro* B lymphopoiesis. BM B progenitor cells (CD10$^+$) were grown on stromal cells in the presence of Wnt3A or medium only for 2 weeks (assay 2). In accordance with the results from the assays using HPC, it was demonstrated on average near 50% reduction in the total number of CD19$^+$ cells in samples treated with Wnt3A compared with control (fig. 4B). When added every third day, both sFRP1 and Dkk1 were able to counteract the effect of Wnt3A almost completely, demonstrating a specific effect of Wnt3A on *in vitro* B lymphopoiesis (fig. 4B). Similar results were obtained using Wnt3A protein from another source; Wnt3A conditioned medium (table 2). Moreover, the effect was independent of the source of stromal cells as the use of primary human BM stromal cells (BMS) as supportive layer did not change the outcome of the experiment (table 2).

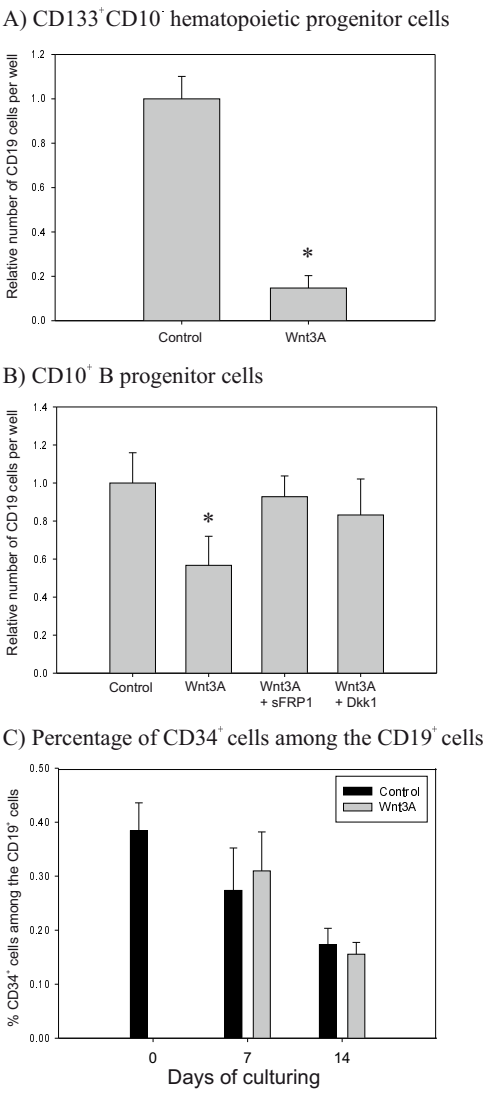

Figure 4

Wnt3A inhibits *in vitro* B lymphopoiesis. BM CD133$^+$CD10$^-$ HPC (A: assay 1) or CD10$^+$ B progenitor cells (B: assay 2) were cocultured with a confluent layer of the murine stromal cell line MS-5 for 3 or 2 weeks, respectively, while treated with Wnt3A (100 ng/ml), Wnt3A + sFRP1 (2 μg/ml), Wnt3A + Dkk1 (500 ng/ml) or medium only. The number of resulting CD19$^+$ B lineage cells in each sample was determined by quantitative flow cytometry. The percentage of CD34$^+$ cells among the CD19$^+$ cells were measured before and after culturing, with and without treatment with Wnt3A (C). The bars represent the mean of N experiments performed in duplicate, ± SEM. A) N = 6. B) Cells treated with control medium or Wnt3A: N = 11, Wnt3A + sFRP1: N = 3, Wnt3A + Dkk1: N = 2. C) day 0: N = 7, day 7: N = 3, Day 14: N = 8. *$p \leq 0.01$, Wilcoxon Signed Ranks Test.

Table 2: Number of CD19 cells after two weeks of culturing BM CD10$^+$ cells on stromal cells

Exp. No. (with MS5)	Control-CM	Wnt3A-CM	Inhibition Index*
1	2182 ± 427	184 ± 91	0.08
2	9440 ± 1953	2652 ± 721	0.28
3	7292 ± 1928	2524 ± 475	0.35
Exp. No. (with BMS)	**Medium**	**rmWnt3A**	**Inhibition Index***
1	1746 ± 300	920 ± 64	0.53

BM CD10$^+$ cells were cultured on a layer of the murine stromal cell line MS-5 in the presence of Wnt3A-conditioned medium (Wnt3A-CM) or control-conditioned medium (control-CM), or on a layer of human bone marrow stromal cells (BMS) in the presence of rmWnt3A or control medium. The numbers in the table represent the mean of duplicate wells ± SD. *Number of CD19$^+$ cells in wells containing Wnt3A divided by number of cells in wells containing control-medium.

To check whether Wnt3A affected distinct early B subpopulations differently, the cells in assay 2 were additionally analyzed for expression of the CD34 differentiation marker to distinguish between pro-B and pre-B cells. The relative frequency of CD34$^+$ cells (pro-B) decreased from 38 % before culturing (day 0), to approximately 30 % and 15 % after one and two weeks of culturing, respectively. This decrease was independent of treatment with or without Wnt3A (fig. 4C). Furthermore, separation of the pre-B population into large cycling and small resting pre-B cells by surface expression of CD20 [33] revealed inhibitory effect of Wnt3A on all subpopulations (results not shown). Thus, we conclude that Wnt3A does not affect the relative proportions of different BM B subpopulations, but has a general inhibitory effect on pro-B, pre-B and immature B cells in a stroma coculture.

Wnt3A inhibits BM B progenitor cell division in vitro

The inhibitory effect of Wnt3A on *in vitro* B lymphopoiesis could be explained by increased apoptosis, an inhibitory effect on proliferation, or both. However, measurements of apoptosis in cells cultured without stromal cells for 1, 2 or 3 days showed no effect of Wnt3A (results not shown), suggesting an effect on proliferation only. To verify this, we used high-resolution cell division tracking to study the initial effects of Wnt3A on B progenitor cells grown on a stromal layer. Sorted CFSE labeled CD10$^+$ B progenitor cells were cocultured with MS-5 for 3 days in the presence of Wnt3A or medium only, and examined for the number of cell divisions by flow cytometry as well as the surface markers CD34 and CD19. The data clearly demonstrated that Wnt3A inhibited the initial divisions of B progenitor cells taking place in the coculture (fig. 5A). When gating for pro-B cells (CD34$^+$CD19$^+$) and pre-B cells (CD34$^-$CD19$^+$) separately, we found that Wnt3A inhibited proliferation of both these populations in a dose-dependent manner (fig. 5B). This effect was blocked by the Wnt antagonist sFRP1 (fig. 5B).

Discussion

Several studies have identified the canonical Wnt pathway as a regulator of the homeostasis of human and murine HSC and hematopoietic progenitor cells [26,34,36]. Furthermore, knockout studies (LEF-1 and Fzd9) in mice have indicated a central role for Wnt signaling in B lymphopoiesis [24,27]. The Wnt pathway also seems to be involved in development of leukemia [28-30]. In the present work, we wanted to study in more detail the implications of canonical Wnt signaling in human BM B lymphopoiesis. Here, we describe that a set of Wnt ligands, Fzd receptors and Wnt antagonists is expressed in BM B progenitor cells, allowing a Wnt signal to be conveyed and modulated in these cells. We demonstrate regulated expression of several Wnt receptors, β-catenin and plakoglobin as well as the transcription factors LEF-1 and TCF-4 mRNAs during early differentiation steps in the B cell lineage, supporting the hypothesis that Wnt signaling is active in BM B lymphopoiesis. Furthermore, we show that canonical Wnt signaling, as measured by the accumulation of β-catenin levels, is induced in human BM B progenitor cells. Finally, we demonstrate that Wnt3A inhibits human stromal dependent B lymphopoiesis and that this effect is a consequence of decreased cell proliferation.

We show that CD10$^+$ human B progenitor cells express a set of Wnt ligand mRNAs (2B, 5B, 8A, 10A and 16), of which Wnt16 is of particular interest, since this gene is activated by the E2A-Pbx1 translocation in some cases of acute lymphocytic leukaemia (ALL) [28]. However, several pre-B leukemia cell lines studied [28] do not express Wnt16, suggesting a distinct role for this factor in early B lymphopoiesis that is turned off during leukemiagenesis, except in cases where Wnt16 is aberrantly activated by the E2A-Pbx1 fusion protein. Further, we demonstrate that primary BM stromal cells express mRNA of several Wnt ligands, including Wnt2B, Wnt5A, Wnt5B, Wnt8B and Wnt9B. This is partly in accordance with previous studies [24,26]. Taken together, these results show that both

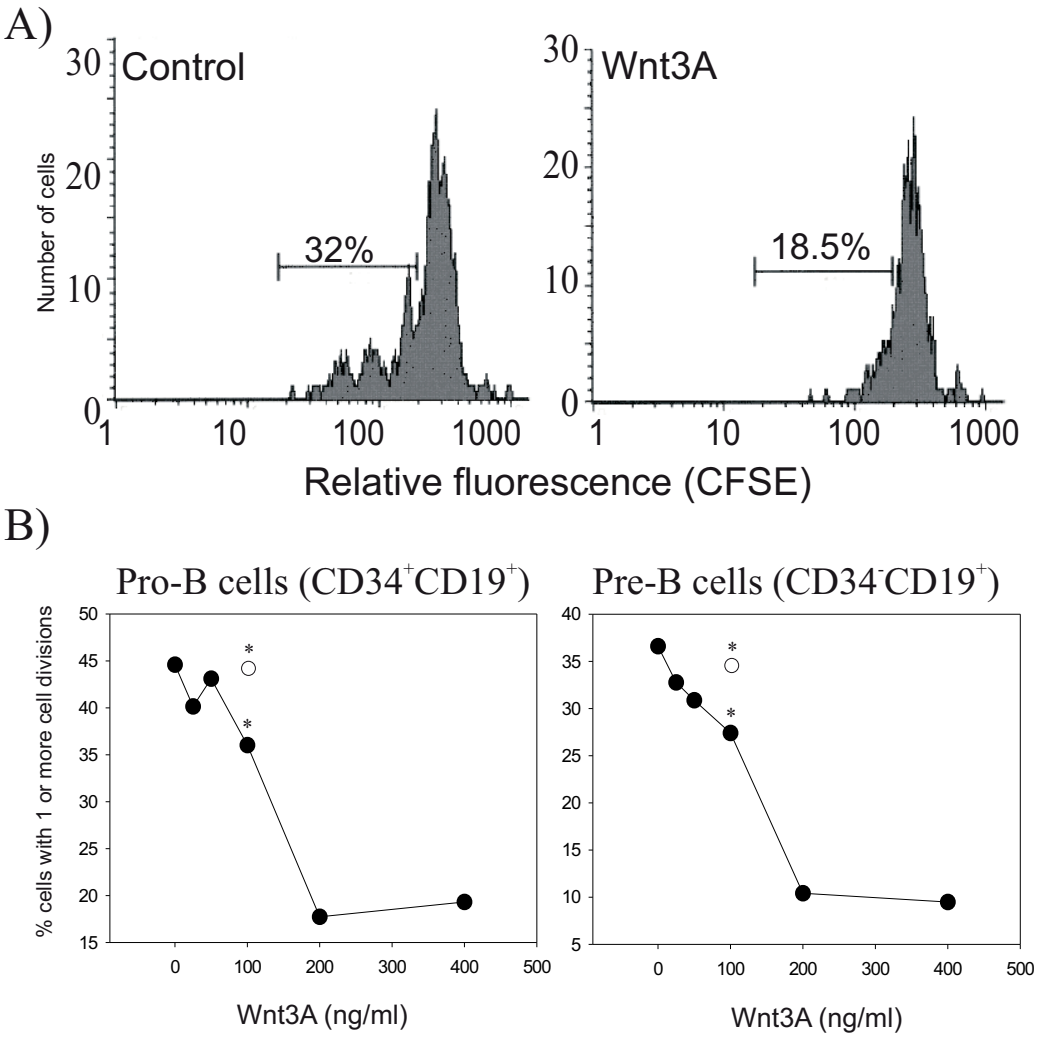

Figure 5

Wnt3A inhibits the initial phase of stromal supported cell division of BM B progenitors. Highly purified BM $CD10^{+}CFSE^{mean}$ cells were grown on a confluent layer of MS-5 and treated with Wnt3A (25–400 ng/ml) or medium only. After three days, the cells were analyzed on a FACScan flow cytometer for the number of cell divisions of $CD19^{+}$ cells. A) Tracking histograms of cell divisions of CFSE-labeled BM B progenitor cells in the presence or absence of Wnt3A (100 ng/ml) One representative experiment of six is shown. B) Dose dependent inhibition of cell division of $CD34^{+}$ pro-B cells and $CD34^{-}$ pre-B cells by Wnt3A (closed circles). The inhibitory effect of Wnt3A was blocked by Wnt antagonist sFRP1 (2 μg/ml) (open circle). Data are shown as percentage of cells that had gone through one or more cell divisions, as determined by cell division tracking with CFSE. One representative experiment of two is shown, except for Wnt3A (100 ng/ml) and Wnt3A + FRP1 (2 μg/ml) where one representative experiment of six is shown (*$p < 0.05$, Wilcoxon Signed Ranks Test, $n = 6$).

hematopoietic cells and the supporting stromal cells may produce Wnt ligands. Different Wnt ligands may have distinct effects during early B lymphopoiesis, which is a topic for future investigations.

So far, only scarce knowledge is available about both ligand specificity and tissue-restricted expression of the Fzd receptors. In our studies we found expression of a wide range of Fzd receptor mRNAs, including Fzd2, 3, 4, 5, 6 and 9, in BM B progenitor cells. Compared to the Wnt mRNAs, these are more readily detectable, indicating higher expression levels, which suggests that Wnt-signaling is important for B progenitor cells. Real-time PCR assays demonstrated differential expression of several Fzd receptor mRNAs, including Fzd5 and Fzd6, which are strongly down-regulated as the cells become immature B cells. Notably, LRP5 and LRP6 mRNAs showed a similar down-regulation. Furthermore, both Fzd5 and Fzd9 are up-regulated as the cells commit to the B cell lineage and go through differentiation. Interestingly, $Fzd9^{-/-}$ mice show a depletion of developing B cells in the BM, particularly in the cycling pre-B population [27]. In contrast to this, our results show that the large cycling pre-B cells express lower levels of LRP5, LRP6, Fzd6, Fzd9, β-catenin and plakoglobin than the small resting pre-B cells. Although one should be cautious in trying to predict functional consequences from mRNA expression data, this trend suggests that Wnt signaling is not likely to be involved in a positive regulation of cycling of the large pre-B cells after Ig heavy chain rearrangement. And even though the absolute expression levels of the receptor mRNAs are low, these data suggest that during a narrow window of the development comprising pro- and pre-B cells, B progenitor cells might be target for Wnt signaling through these receptors.

To be able to convey a Wnt-signal, the cells have to express either of the two important molecules, β-catenin or plakoglobin. Our results show that levels of β-catenin mRNA change little during the differentiation. Although it has been demonstrated that levels of cytoplasmic β-catenin protein may vary throughout the development of thymocytes [37], these variations may not necessarily be reflected by the mRNA levels. In fact, as β-catenin is needed both for signaling purposes as well as for adhesion purposes, the mRNA levels may have to be kept relatively stable. Plakoglobin mRNA, on the other hand, decreases after the pro-B differentiation level. This corresponds to the observations made in developing murine thymocytes [37], where plakoglobin is down-regulated at the level of immature single positive thymocytes, suggesting that plakoglobin may play a central, but hitherto unexplored role in conveying a Wnt signal during lymphopoiesis. In fact, the lack of effect of knocking down β-catenin in early hematopoiesis, including B and T lymphopoiesis [38], prompted the authors to suggest that plakoglobin may stand-in for β-catenin in this respect.

The LEF-1/TCFs are directly activated by canonical Wnt signaling, and LEF-1 knockout mice show defects in pro-B cell proliferation and survival [24]. However, it cannot yet be ruled out that this effect might be a result of abolishment of the repressive functions or other non-Wnt related activities of LEF-1 [15]. Here, we have verified microarray data showing regulation of LEF-1 and TCF-4 during B lymphopoiesis (Hystad ME *et al*, manuscript in preparation and [33]). Interestingly, it has been reported that LEF-1 is a target gene for the B lymphopoiesis key transcription factor Pax-5 [39]. Moreover, LEF-1 interacts with Pax-5 and c-Myb to activate the Rag-2 promoter [40], but the accurate role of LEF-1 in B lymphopoiesis is still elusive. In contrast to LEF-1, we found TCF-4 mRNA levels to be high in ELP and pro-B cells, and lower in the more mature pre-B and immature B populations. Although expressed at lower levels, one could speculate that TCF-4 steps in for LEF-1 in the earliest lymphoid progenitors before LEF-1 is properly switched on, potentially in conveying a Wnt signal or, alternatively, in acting as a transcription repressor of B lineage genes before commitment. These are topics for further studies.

Wnt antagonists play important roles in preventing or fine tuning the Wnt signal [16]. Our data show expression of the Wnt antagonists Dkk1, Dkk4, sFRP4 and WIF1 mRNAs in B progenitor cells. Dkk1, sFRP2 and sFRP3 were expressed in bone marrow stromal cells. Of these factors, Dkk1 in particular is known to be involved in a feedback loop to adjust or shut down canonical Wnt signaling [41]. It is likely that these factors are important in adjusting the incoming Wnt signals in the bone marrow microenvironment, where several cell types are able to express a wide range of ligands and Wnt receptors.

The inhibitory effect of Wnt3A on the generation and cell division of B progenitor cells *in vitro*, both with regard to pro- and pre-B cells, is in contrast to several reports on the functional effects of canonical Wnt signaling in mice. Both in murine HSC [34], developing thymocytes [25] and a wide range of cancer cells [31,42], elevated levels of β-catenin lead to increased cell proliferation. Furthermore, in fetal murine pro-B cell [24], Wnt3A conditioned medium leads to increased BrdU incorporation. Our divergent results may be due to different species, microenvironments and/or cell context. For instance, murine and human B lymphopoiesis require to a certain extent differing factor dependency [32]. However, by culturing murine BM B progenitor cells, we have not been able to demonstrate increased cell proliferation in the presence of Wnt3A (results not shown). Thus, we suspect the Wnt response to be different in fetal and adult B progenitor

cells, potentially affected by the cellular microenvironment and/or context. Indeed, the fetal pro-B cells are exposed to the microenvironment of the liver and this is very different from that of the BM. For instance several regulators of the Wnt pathway are more highly expressed in fetal liver stroma than in BM stroma [43], which suggest that Wnt signaling might be regulated in a different manner and have a different role in the fetal liver than in the BM. Another important aspect that has to be taken into consideration, is that different Wnt ligands, although able to activate canonical Wnt signaling, indeed show distinct activities [44]. In addition there may also be species and location differences. However, as mentioned above, Cobas *et al* have demonstrated a lack of an essential role for β-catenin in BM hematopoiesis, including proliferation of B lymphocytes [38]. Thus, in contrast to findings in the fetal liver, our results may very well represent a physiological situation in the adult organism, where Wnt signaling via β-catenin is not essential for B lymphocytes, but may be used to fine tune the delicate balance between proliferation, differentiation and apoptosis taking place during early BM B lymphopoiesis.

In support of our data on an inhibitory effect of Wnt3A on cell division, it has been reported that canonical Wnt signaling hampers fibroblast cell proliferation through cell cycle blocks, potentially mediated via p53 [45]. Moreover, Wnt signaling inhibits proliferation and regulates cell-cycle arrest at distinct stages of development in Drosophila wing development [46]. Thus, it is likely that the cellular context, in some cases represented by the ability of a central regulatory molecule like p53 to respond, will affect how the cells react to vital stimuli like Wnt. It has been speculated that aberrant p53 is necessary to convey the strong tumor promoting effect of abnormal Wnt signaling seen in colon cancer [47,48]. It is also interesting that Wnt5A has been found to inhibit B cell proliferation and can function as a tumor suppressor in hematopoietic tissue, albeit via the non-canonical Wnt/Ca^{2+} pathway [49].

We show expression of Wnt2B, 5B, 8A, 10A and 16 in BM $CD10^+$ cells and of Wnt2B, Wnt5A, Wnt5B, Wnt8B and Wnt9B mRNAs in human primary BMS cells. Further we demonstrate that Wnt3A acts directly on B progenitor cells by increasing the levels of β-catenin, suggesting that the microenvironment may use Wnt signaling to regulate the fate of developing B lymphocytes. Yet, we cannot exclude that the functional effect of Wnt3A on *in vitro* B lymphopoiesis is indirect and mediated via the stromal cells, as observed for *in vitro* hematopoiesis [35]. The BM microenvironment is composed of a heterogeneous population of cells including fibroblasts, adipocytes, endothelial cells and osteoblasts, all derived from a common mesenchymal precursor [50]. In particular, the role of Wnt signaling in adipogenesis may be relevant here, as it has been demonstrated that Wnt10B [51,52] inhibits adipogenesis, and there seems to be a positive correlation between adipogenesis and hematopoiesis [52]. This emphasizes the complexity of the interactions in the B lymphopoiesis maturation niche and opens for the possibility that B progenitor cells may manipulate the stromal support via these Wnt factors. However, it is not uncommon in developmental niches that morphogenic signals have the potential to act on several cells in the microenvironment. Therefore, it has been suggested that Wnt signaling might influence the HSCs both directly and indirectly by maintenance of the cellular elements of the stem cell niche [21]. In line with this theory, several studies have demonstrated expression of multiple Wnt mRNAs in thymocytes and the thymic microenvironment. It is likely that particular Wnts serve distinct roles, thus, cell specific effects may be achieved by "playing the Wnt repertoire" as well as through combinations with other signaling events.

Conclusion

In this study, we have demonstrated mRNA expression of several Wnt ligands, Fzd receptors and Wnt antagonists in human BM B progenitor cells and regulated expression of Fzd receptors and co-receptors, β-catenin, plakoglobin, LEF-1 and TCF-4 mRNA in these cells during differentiation. Furthermore, we find that Wnt3A induced an accumulation of β-catenin in the BM B progenitor cells and inhibition of *in vitro* B lymphopoiesis. These results suggest the Wnt/β-catenin pathway as a negative regulator of human stromal dependent B lymphopoiesis. This is in contrast to observations on Wnt effects in fetal murine pro-B cells, and may represent a distinction between the fetal liver and adult BM microenvironments.

Methods

Reagents and antibodies for FACS and western blot analysis

Recombinant murine (rm) Wnt3A, recombinant human (rh) secreted frizzled related protein 1 (sFRP1), rh Dickkopf 1 (Dkk1), rh interleukin (IL)-7, rh IL-3 and rh Flt3 ligand (FL) were purchased from R&D Systems (Great Britain). The following monoclonal antibodies (mAbs) were used for flow cytometry: anti-CD34 PE, anti-CD10 APC, anti-CD10 PE-Cy7 and anti-CD20 APC from Becton Dickinson, Biosciences Pharmingen (San Jose, CA, USA), anti-CD19 PE-Cy5 and anti-CD34 PE-Cy5 from Immunotech (Marseille, France) and anti-CD19 PE, anti-CD45 PE and anti-IgM Fitc from Dako Cytomation (Denmark). Irrelevant isotype matched Abs were used as controls. The following Abs were used in western blot analysis: anti-β-catenin (Mouse IgG1, BD Transduction Laboratories), anti-β-actin (Goat polyclonal IgG, Santa Cruz Biotechnology), rabbit anti-mouse IgG1-HRP and rabbit anti-goat IgG-HRP (Dako cytomation, Denmark).

Primary cells and cell lines

BM aspirates were from the iliac crest of normal adult volunteers (approved by the Regional Ethical Committee). Mononuclear cells (MNC) were separated by Ficoll-Hypaque density gradient centrifugation (Lymphoprep, Nycomed, Norway). $CD10^+$ B progenitor cells (ELP, pro-B and pre-B cells) were isolated from BM MNC using Dynabeads®M-450 Epoxy (Dynal, Oslo, Norway) directly coated with anti-CD10 mAb (clone RFAL-3, Sigma-Aldrich, UK) followed by detachment using CD4/CD8 DETACHaBEAD (Dynal, Norway) according to the producer 's protocol. The $CD10^+$ cells were of 90–95% purity, they were $CD45^+$ and contained 4–7% IgM^+ cells (immature B cells). $CD34^+$ and $CD19^+$ cells were isolated in a similar manner from MNC using Dynabeads®M-450 conjugated with anti-CD34 or anti-CD19 mAb, respectively, and CD34 or CD19 DETACHaBEAD (Dynal, Norway), respectively. $CD133^+CD10^-$ cells (HPC) were isolated from the $CD10^-$ fraction of BM MNC (see above) using the MACS system (Magnetic cell sorting of human cells) and a CD133 Cell Isolation Kit (Miltenyi Biotec, Germany). Briefly, the mononuclear cells were magnetically labeled with CD133 MicroBeads and separated on a column, which was placed in the magnetic field of a MACS Separator. The magnetically labeled $CD133^+$ cells were retained in the column while the unlabeled $CD133^-$ cells passed through. After removal of the column from the magnetic field, the magnetically retained $CD133^+$ cells were eluted as the positively selected cell fraction. The $CD133^+$ cells were typically of 97–98% purity. In monoculture, the cells were kept in X-VIVO 15™ (BioWhittaker, Walkersville, USA) with 0.1% detoxified BSA.

The murine stromal cell line MS-5 [53] was cultured in α-MEM with 10% FCS and 100 μg/ml of penicillin and streptomycin (PAA Laboratories, Pasching, Austria) and was passaged twice a week. Cultures of human BM stromal (BMS) cells were established as previously described [54]. Briefly, total BM MNC cells depleted of $CD34^+$ cells were seeded into 75-cm^2 tissue culture flasks in RPMI-1640 with 10% FCS, penicillin and streptomycin. Non-adherent cells were washed off after 2 hours at 37°C, and the adherent cells were cultured in EX-CELL 610 (JRH Biosciences, USA) with 10% FCS, penicillin and streptomycin. The BMS cells were passaged twice before they were used for experiments.

The human ALL cell lines Reh (no ACC 22, DSMZ) and Nalm-6 (no ACC 128, DSMZ) (Hurwitz et al, 1979) were kept in X-VIVO 15™ supplemented with 100 μg/ml of penicillin and streptomycin.

FACS analysis and cell sorting

Cells were stained with anti-CD19 PE Ab for 30 min at 4°C before analysis on FACSCalibur flow cytometer (argon-ion laser tuned at 488 nm; Becton Dickinson). Quantitative analysis of $CD19^+$ cells in cocultures was performed using Flow Cytometry Absolute Count Standard, from Bangs Laboratories Inc., (Fishers, IN 46038 USA). Data acquisition and analysis were performed using CELLQuest software (Becton Dickinson).

Highly purified (98–99%) B progenitor cells for RT-PCR analysis of Wnt, Fzd and Wnt antagonist mRNA expression were obtained by sorting of BM $CD10^+CD45^+IgM^-$ B progenitor cells using a FACSDiVa flow cytometer (Becton Dickinson, USA) after staining of BM $CD34^+$ and $CD19^+$ isolated cells with anti-CD45 PE, anti-CD10 APC and anti-IgM FITC Abs. Highly purified BM cell populations for real-time PCR were obtained by staining BM $CD34^+$ and $CD19^+$ cells with anti-CD10 PE-Cy7, anti-CD34 PE, anti-CD19 PE-Cy5, anti-CD22 APC and anti-IgM Fitc Abs and the following subpopulations were sorted using a FACSDiVa flow cytometer: ELP ($CD10^+CD34^+CD19^-IgM^-$), pro-B ($CD10^+CD34^+CD19^+CD20^-IgM^-$), large pre-B ($CD10^+CD34^-CD19^+CD20^{dim}IgM^-$), small pre-B ($CD10^+CD34^-CD19^+CD20^-IgM^-$) and immature B ($CD10^+CD34^-CD19^+CD20^{high}IgM^+$). Separation of large and small pre-B cells was based on both CD20 expression and size (forward scatter, FSC).

PCR analysis

Total RNA from freshly isolated and sorted BM $CD45^+CD10^+IgM^-$ cells was isolated using Absolutely RNA™ RT-PCR Mini-prep kit (Stratagene Europe, Amsterdam, Netherland) according to the manufacturer's instructions. RNA from human fetal brain was purchased from BioChain Institute, Inc., USA. cDNA was synthesized from 1 μg total RNA primed with random hexamers in a 50 μl reaction using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Control reactions lacking reverse transcriptase were always included. RT-PCR of 20 ng of total RNA was performed with a titanium polymerase (BD Biosciences, USA) in a 25 μl reaction for 37 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 68°C for 30 seconds. The primer sequences used to identify Wnt, Fzd and Wnt antagonist gene expression are listed in Table 3. The primer sequences was partly designed specifically for this work and partly copied from previous expression analyses [55]. For all mRNAs expressed, the amplified products have been sequenced and confirmed to represent the correct target gene.

Real-time PCR

Total RNAs from 5–20 000 freshly isolated and sorted BM B progenitor cells (ELP, pro-B, large pre-B, small pre-B and immature B cells) were purified using Absolutely RNA™ RT-PCR Micro-prep kit (Stratagene Europe, Amsterdam, Netherland) according to the manufacturer's instructions.

Table 3: Primer sequences used for mRNA expression analyses of Wnt ligands, Fzd receptors and Wnt antagonists

Primer	*Sequence*	*Amplicon (bp)*
Wnt1	F-5' TAG CCT CCT CCA CGA ACC TG-3'	239
	F-5' CAG CCT CGG TTG ACG ATC TTG-3'	
Wnt2	F-5' TGG TGG TAC ATG AGA GCT ACA GGT G-3'	297
	R-5' CCC TGG TGA TGG CAA ATA CAA C-3'	
Wnt2B	F-5' TCA TGC TCA GAA GTA GCC GAG A -3'	328
	R-5' TGG CAC TTA CAC TCC AGC TTC A -3'	
Wnt3	F-5' CTG GCT ACC CAA TTT GGT GGT-3'	225
	R-5' CAT CTA TGG TGG TGC AGT TCC A-3'	
Wnt3A	F-5' AAG CAG GCT CTG GGC AGC TA-3'	234
	R-5' GAC GGT GGT GCA GTT CCA-3'	
Wnt4	F-5' GAG GAG ACG TGC GAG AAA CTC AA-3'	346
	R-5' ATC CTG ACC ACT GGA AGC CCT GT-3'	
Wnt5A	F-5' ATC CTG ACC ACT GGA AGC CCT GT-3'	358
	R-5' GGC TCA TGG CGT TCA CCA C-3'	
Wnt5B	F-5' CAG CTT CTG ACA GAC GCC AAC T-3'	279
	R-5' GCC TAT CTG CAT GAC TCT CCC A-3'	
Wnt6	F-5' GCT CCA GCC ACA GCA AGG-3'	378
	R-5' CAG CCT GCC CGC CTC GTT-3'	
Wnt7A	F-5' CCT GGG CCA CCT CTT TCT CAG-3'	573
	R-5' TCC AGC TTC ATG TTC TCC TCC AG-3'	
Wnt7B	F-5' TTT CTC TGC TTT GGC GTC CT-3'	391
	R-5' TGG TTG TAG TAG CCC TGC TTC TC-3'	
Wnt8A	F-5' TCC CAA GGC CTA TCT GAC CTA C-3'	400
	R-5' CCG GCC CTG TTG TTG TGA-3'	
Wnt8B	F-5' GCC CAG AGT GGT ATT GAA GAA TG-3'	266
	R-5' TTG TCA CTG CAG CCT CCC-3'	
Wnt9A	F-5' AAG TAC AGC AGC AAG TTC GTC AAG G-3'	538
	R-5' GCA CTC CAC ATA GCA GCA CCA AC-3'	
Wnt9B	F-5' AGT TTC AGT TCC GGC ATG AGC-3'	340
	R-5' TTC ACA GCC TTG ATG CCC A-3'	
Wnt10A	F-5' ACA CAG TGT GCC TAA CAT TGC C-3'	296
	R-5' AGG CCT TCA GTT TGC CCA G -3'	
Wnt10B	F-5' CCT CGC GGG TCT CCT GTT C-3'	563
	R-5' GGT TAC AGC CAC CCC ATT CC-3'	
Wnt11	F-5' ACA ACC TCA GCT ACG GGC TCC T-3'	394
	R-5' CCC ACC TTC TCA TTC TTC ATG C-3'	
Wnt16	F-5' CTG TGC AAG AGG AAA CCG TAC CTG-3'	520
	R-5' CAG CAC AGG AGC CGG AAA CT-3'	
Fzd1	F-5' CTC TAC TTC TTC AGC ATG GCC A-3'	230
	R-5' TCC ACG TTG TTA AGC CCC A-3'	
Fzd2	F-5' CCA TCC TAT CTC AGC TAC AAG TTT CT-3'	306
	R-5' GCA GCC CTC CTT CTT GGT-3'	
Fzd3	F-5' TCC CCT CTG CCT GTA TGT GGT AGT-3'	622
	R-5' GCT GCT CAC TTT GCT GTG GA-3'	
Fzd4	F-5' CTC GGC TAC AAC GTG ACC AAG AT-3'	605
	R-5' AAT ATG ATG GGG CGC TCA GGG TA-3'	
Fzd5	F-5' GTG CCC ATT CTG AAG GAG TCA C-3'	197
	R-5' TCC ATG TCG ATG AGG AAG GTG-3'	
Fzd6	F-5' ACT CTT GCC ACT GTG CCT TTG-3'	300
	R-5' GTC GAG CTT TTG CTT TTG CCT-3'	
Fzd7	F-5' CAA GAC CGA GAA GCT GGA GAA G-3'	248
	R-5' TGC CGA CGA TCA TGG TCA T-3'	
Fzd8	F-5' GGA CTA CAA CCG CAC CGA CCT-3'	407
	R-5' ACC ACA GGC CGA TCC AGA AGA C-3'	
Fzd9	F-5' TCA AGG TCA GGC AAG TGA GCA-3'	210
	R-5' AGC TTC CAG AGG AAC GCA ACA-3'	
Fzd10	F-5' CAG GTG TGC AGC CGT AGG TTA A-3'	212
	R-5' AAG CAC CAC ATC TTA GCT CCG G-3'	
WIF1	F-5' ACG GAC CTC ACT GTG AGA AAG C-3'	200

Table 3: Primer sequences used for mRNA expression analyses of Wnt ligands, Fzd receptors and Wnt antagonists *(Continued)*

	R-5' GCT GAT TTC ACA CTG CTC TCC C-3'	
sFRP1	F-5' GGT CAT GCA GTT CTT CGG CT-3'	206
	R-5' TCC TCA GTG CAA ACT CGC TG-3'	
sFRP2	F-5' ACC GAG GAA GCT CCA AAG GTA T -3'	259
	R-5' TCA TCT CCT CAC AGG TGC ACT G -3'	
sFRP3	F-5' CTC ATC AAG TAC CGC CAC TCG TG-3'	210
	R-5' CCG GAA ATA GGT CTT CTG TGT AGC TC-3'	
sFRP4	F-5' GCA CAT GCC CTG GAA CAT CAC-3'	243
	R-5' ATC TTC ATG AGG GGC TCG CAG T-3'	
Dkk1	F-5' ACC ATT GAC AAC TAC CAG CCG T -3'	235
	R-5' TGG TTT CCT CAA TTT CTC CTC G -3'	
Dkk4	F-5' CGT TCT GTG CTA CAT GTC GTG G-3'	241
	R-5' TCT TGT CCC TTC CTG CCT TGT-3'	

cDNAs were synthesized from total RNA primed with random hexamers using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). LEF-1 and TCF-4 (gene name TCF-7L2) mRNA expression was analyzed by real-time quantitative RT-PCR using Taqman technology according to the manufacturers procedure (Applied Biosystems). Predeveloped assay reagents including primers and probes for LRP5 (Hs00182031_m1), LRP6 (Hs00233935_m1), Fzd2 (Hs00361432_s1), Fzd5 (Hs00361869_g1), Fzd6 (Hs00171574_m1), Fzd9 (Hs00268954_s1), β-catenin (CTNNB1, Hs00170025_m1), plakoglobin (JUP, Hs00158408_m1), LEF-1 (Hs00212390_m1) and TCF-4 (Hs00181036_m1) mRNAs as well as the endogenous control phosphoglycerate kinase 1 (PGK1) (Hs99999906_m1) were supplied by Applied Biosystems and the PCR reactions were performed according to the manufacturer's instructions using Taqman Universal PCR Master Mix. Each measurement was performed in duplicate and the expression level for each gene was calculated using the standard curve method for relative quantitation of gene expression as described by the manufacturer (ABI Prism 7700 Sequence Detection System, User Bulletin 2, PE Applied Biosystems, Foster City, CA). Total RNA from the ALL cell lines Reh and Nalm6 as well as total RNA from human fetal brain were used for standard curves. Expression values for PGK1 mRNA, initially determined to be a suitable endogenous control for BM populations, were used for normalization of the expression levels. The expression level of the different genes in pro-B cells was used as a calibrator, and the expression of the other populations were calculated relative to the expression in pro-B cells.

Western blot analysis

The cells were treated with Wnt3A or vehicle only (PBS with 0.1% detoxified BSA) for 3 hours and total cell lysates were analyzed by Western blot using 10% SDS polyacrylamide gels from Pierce (Rockford, USA) as described earlier [56]. The filters were pretreated with PBS containing 0.1% Tween-20 (PBS-T) and 5% dry milk, incubated overnight with anti-β-catenin Ab or 1 hour with anti-β-actin Ab and then washed 2 × 15 min in PBS-T. The filters were then incubated with the secondary Ab rabbit anti-mouse IgG1-HRP Ab or rabbit anti-goat IgG-HRP Ab, respectively, for 60 minutes at room temperature and washed 2 × 15 min in PBS-T before the proteins were visualized using ECL+ Western Blotting Detection Reagents from Amersham Biosciences (Piscataway, NJ, USA).

Hematopoietic cell-stromal cell coculture

Assay 1: HPC ($CD133^+CD10^-$) were cultured in 24 well tissue plates (2000 cells/well) pre-seeded with MS-5 (2.5×10^4 cells/well). Assay 2: B progenitor cells ($CD10^+$) were cultured in 96 well tissue plates (8000 cells/well) pre-seeded with MS-5 (1×10^4 cells/well). Both sets of cocultures were in α-MEM containing 1% FCS, 100 μg/ml of penicillin and streptomycin, and supplemented with cytokines (for HPC: SCF, 25 ug/ml and G-CSF, 2,5 ug/ml, for B progenitor cells: IL-7, 50 ng/ml, IL-3, 20 ng/ml and FL, 50 ng/ml). In one additional experiment, the wells were pre-seeded with BMS (1×10^4 cells/well) in EX-CELL 610 with 1% FCS, 100 μg/ml of penicillin and streptomycin and cytokines (IL-7, 50 ng/ml, IL-3, 20 ng/ml and FL, 50 ng/ml). Where indicated, Wnt3A (10–100 ng/ml), Dkk1 (500 ng/ml) or sFRP1 (2 μg/ml) were added to the cultures. 50% of the medium was replaced weekly. After 3 (HPC) or 2 (B progenitor cells) weeks of culturing, single wells were harvested by trypsination and the B progenitor cells were immunophenotyped using the pan B cell marker CD19 as well as the CD34 differentiation marker and subjected to quantitative analyses (see above). Wnt3A conditioned medium and control medium were collected from L-Wnt3A cells and control nontransfected L-cells, respectively (purchased from American Type Culture Collection (ATCC), Manassas, USA), according to the manufacurer's procedure.

High-resolution cell division tracking

BM $CD34^+$ and $CD19^+$ cells were labeled with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) as described earlier [57]. To allow unbound dye to diffuse from cells, labeled cells were seeded on a confluent layer of MS-5 and incubated for 18–24 hours at 37°C in α-MEM with 1% FCS. Subsequently, the cells were stained with CD10 APC mAb and $CD10^+CFSE^{mean}$ cells were sorted on a BD FACSDiVa flow cytometer (Becton Dickinson). Sorted cells (1.5–2 × 10^4/well) were cultured in 48 well tissue plates pre-seeded with MS-5 (2 × 10^4 cells/well) supplemented with IL-7 (50 ng/ml) and FL (50 ng/ml) and treated with Wnt3A (25–400 ng/ml), Wnt3A + sFRP1 (2 μg/ml) or medium only. IL-3 was left out of these cultures, because earlier experiments showed that IL-7 and FL were sufficient to support survival and proliferation of the B progenitor cells (data not shown). After three days the cells were harvested by trypsination and analyzed on a FACSCalibur flow cytometer for the number of cell divisions as well as expression of the cell surface markers CD34 and CD19.

Statistical analysis

The statistical significance of differences between groups was determined using the paired two-tailed Wilcoxon's nonparametric test, by applying SPSS 11.5 software.

Abbreviations

Wnt, Wingless-type MMTV integration site family

BM, bone marrow

Fzd, Frizzled

HSC, hematopoietic stem cell

HPC, hematopoietic progenitor cell

CLP, common lymphoid progenitor

ELP, early lymphoid progenitor

IL-7, interleukin 7

IL-3, interleukin 3

FL, Flt3 ligand, Fms-related tyrosine kinase 3 ligand

SDF-1, stromal cell-derived factor 1

Dkk, Dickkopf

sFRP, soluble Fzd related protein

GSK3β, glycogen synthase kinase-3β

LRP, low density lipoprotein receptor-related protein

LEF-1, lymphoid enhancer-binding factor 1

TCF-4, transcription factor 4

WIF1, Wnt inhibitory factor 1

CFSE, carboxyfluorescein diacetate succinimidyl ester

ALL, acute lymphocytic leukaemia

CLL, chronic lymphocytic leukaemia

BSAP, B-cell lineage specific activator protein

Pax-5, paired box gene 5

Rag-2, Recombination-Activating Gene-2

Rm, recombinant murine

Rh, recombinant human

mAb, monoclonal antibody

MNC, mononuclear cells

BMS, bone marrow stroma

BSA, bovine serum albumin

PGK1, phosphoglycerate kinase 1

Authors' contributions

GD designed and conducted experiments, oversaw research, and wrote paper. ET designed and conducted experiments, oversaw research, and wrote paper. MKN designed and conducted experiments, oversaw research. HS designed and conducted experiments. SF designed experiments, oversaw research, and wrote paper. ER designed experiments, oversaw research, and wrote paper.

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