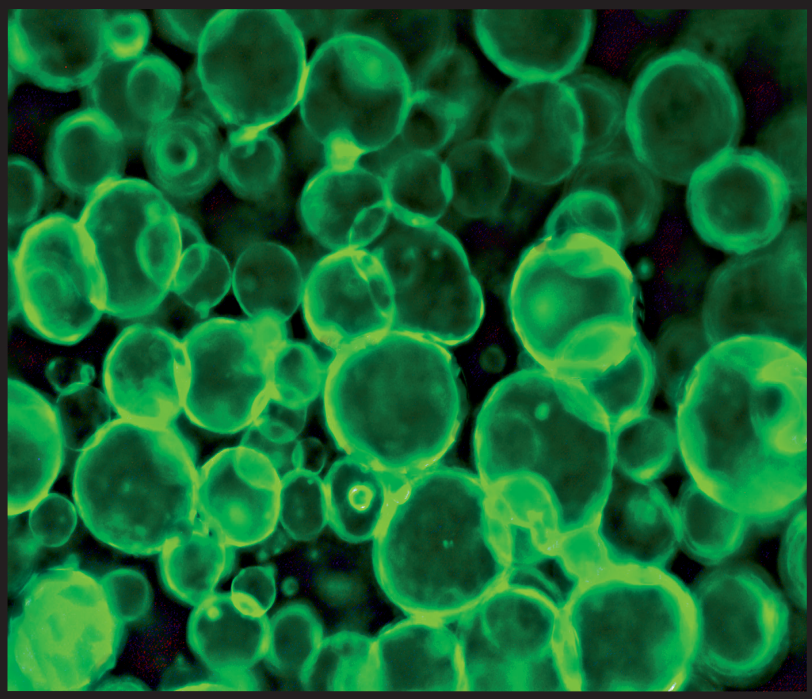
# Effects of Wnt Signaling Proteins on Maintenance and Expansion of Adult Stem Cells



Nesrin Tuysuz

# Effects of Wnt Signaling Proteins on Maintenance and Expansion of Adult Stem Cells

**Nesrin Tüysüz**

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*Hayat kısa, kuşlar uçuyor*

*(Cemal Süreya)*

# Effects of Wnt Signaling Proteins on Maintenance and Expansion of Adult Stem Cells

Effecten van Wnt signaal eiwitten op onderhoud
en expansie van somatische stamcellen

**Thesis**

to obtain the degree of Doctor from the
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born in Istanbul, Turkey

Erasmus University Rotterdam

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## Scope of the thesis

Adult tissue homeostasis and repair relies on a small population of stem cells residing among the differentiated cells of the respective tissues. Adult stem cells are defined by their capacity to renew themselves (self-renewal) and to generate all the differentiated cell types of the tissue (multipotency). These two abilities render adult stem cells very attractive for therapeutic purposes, because they might restore lost, damaged, or aging cells and tissues in the human body. With respect to this, hematopoietic stem cell transplantation has achieved significant therapeutic success over the years. Moreover, the recent establishment of stem cell cultures from intestine, liver and other organs in organoids holds great promise for personalized and regenerative medicine. Nevertheless, several restrictions of the stem cell based therapeutic approaches such as the shortage of compatible donors, low number of stem cells and undefined culture conditions limit their widespread use. Therefore, the ability to propagate adult stem cells in defined conditions *ex vivo* would undoubtedly accelerate the exploitation of these stem cells for clinical purposes. An understanding of the cues governing the decisions between self-renewal and differentiation is therefore necessary. The Wnt signaling pathway is a key signaling cascade implicated in control of stem cell self-renewal and differentiation from a variety of mammalian tissues including blood, intestines, hair follicles, and liver. Therefore, purified Wnt proteins serve as important tools to regulate stem cell behavior *in vitro*. The work described in this thesis aims to investigate the effects and probable use of Wnt signaling proteins in propagation of adult stem cells from a variety of tissues, including intestines, blood and liver, for improving the applicability of adult stem cells in clinics.

To provide the necessary background information relevant to the work described in this thesis, the **Chapter 1** is dedicated to a general introduction. The first part of this chapter describes the adult stem cell systems from blood, intestine and liver. This will be followed by the part dedicated to the Wnt signaling pathway and its roles in adult stem cell systems. In the last part, the applicability of liver stem cells in gene therapy of lysosomal storage disorders with the focus of Hurler disease will be explained.

The derivation and expansion of stem cells in the form of organoids from human intestine and liver are dependent on Wnt signals provided in the form of a Wnt3a conditioned-medium, which contains serum and other undefined factors. The use of stem cells in clinics, however, requires a robust and defined cell culture medium. **Chapter 2** describes how exploiting the hydrophobic nature of Wnt proteins to stabilize them using lipid-modified nanoparticles instead of detergents enables the establishment of human intestine and liver organoids in serum-free media.

The Wnt signaling pathway has been implicated to play a role in the regulation of hematopoietic stem cell self-renewal and differentiation. **Chapter 3** and **4** address the effect of Wnt3a induced signaling in *ex vivo* culture of hematopoietic stem and progenitor cells from mouse bone marrow and human umbilical cord blood, respectively.

Having established the serum-free culture of adult liver organoids, we describe in **Chapter 5** a proof-of-principle study investigating the use of these stem cells for gene therapy of lysosomal storage disorders, in particular Hurler syndrome.

Finally, in **Chapter 6** the significance and implications of the studies performed in this thesis are discussed, and directions for further studies are provided.

# CHAPTER 1

# General introduction

## ADULT STEM CELLS

### From the cell theory to the modern stem cell concept

The invention of the microscope at the beginning of the seventeenth century enabled the observation of microscopic life for the first time. In 1665, using the microscope he devised Robert Hooke analyzed the structural units of a slice of cork and coined the term 'cells' to refer to these units. Although Hooke used this term differently, the word 'cell' that we use today comes directly from his work. A few years later, another distinguished microscopist of the time, Antonie van Leeuwenhoek, observed and described unicellular living organisms, which he named as 'animalcules' for the first time. Van Leeuwenhoek also studied human semen and discovered spermatozoa. After Leeuwenhoek, however, it took two hundred years to improve microscopes as well as tissue preservation techniques for further key observations. For instance, in the first half of the 19$^{th}$ century, Karl Ernst von Baer finally proved the presence of the mammalian egg under the microscope. Around the same time, Matthias Jakob Schleiden and Theodor Schwann proposed that all plant and animal tissues were composed of cells. Later on, Remak, Virchow and Kölliker showed that cells arise from pre-existing cells. All together, these findings led to one of the most important generalizations of biology, the 'cell theory', stating that all organisms are made of cells which are the basic units of life and which arise from a pre-existing cell[1].

With the advent of the cell theory, it was realized that egg and sperm were also cells. Soon after, the fertilization in sea urchins was demonstrated by Oscar Hertwig and the big question of how does a multicellular embryo develop from a single fertilized egg emerged[2]. At the time, Hertwig was a student of Ernst Haeckel, a major supporter of Darwin's theory of evolution who was particularly interested in common ancestors (phylogeny) along with embryology (ontogeny). In his work, Haeckel coined the term 'Stammzellen' (german for stem cells) and used it in two senses: as the common unicellular ancestor to multicellular organisms as well as the fertilized egg giving rise to all cells of the organism. The term 'stem cell' reappeared, a few years later in the late 19$^{th}$ century, when several scientists including Theodor Boveri and Valentin Häcker used it to describe the cells giving rise to the primordial germ cells as well as primordial somatic cells. At the same time, several histologists such as Alexander Maximow, Ernst Neumann and Artur Pappenheim were starting to use this term for a common precursor of the blood system[3]. However, the phrase 'stem cells' remained an abstract definition until the demonstration of the existence of hematopoietic stem cells (HSCs) with a series of seminal experiments by James Till, Ernest McCulloch and others in the 1960s. Till and McCulloch first demonstrated that a subset of bone marrow cells could form macroscopic colonies containing differentiated progeny of multiple blood lineages[4,5]. A subset of these colonies, when transplanted into secondary hosts, could reconstitute all the blood lineages[6,7]. With these discoveries, the stem cell concept has gained its operational definition: stem cells are cells, which can proliferate indefinitely (self-renewal) and give rise to one or more specialized cell types (potency).

The stem cell concept has ever since vastly expanded owing to studies ranging from the early embryo to adult organisms. In 1981, Martin, Evans and Kaufman described the isolation of cells from the inner cell mass of the mouse blastocyst for the first time[8,9]. These cells, referred to as embryonic stem cells (ESCs), display an indefinite self-renewal capacity *in vitro* under proper culture conditions and retain the ability, known as pluripotency, to develop into all cell types of the embryo proper. On the contrary to pluripotent ESCs,

adult stem cells, which reside in postnatal tissues (e.g. bone marrow[10], liver[11], intestine[12]), are multipotent, meaning that they are capable of specializing into the cell types of the respective tissue but not all cell types of the organism. Rare populations of multipotent adult stem cells, with their ability to self-renew, play essential roles in the maintenance and repair of adult tissues. Therefore, adult stem cells are also very valuable for therapeutic purposes. Today, hematopoietic stem cell transplantation is routinely used in clinical applications for treatment of a variety of hematological diseases[13]. Moreover, recent advances in culture of many other adult stem cell types including stomach, intestines and liver offer many promises to the field of regenerative medicine[14–16]. Nonetheless, much remains to be answered about different types of adult stem cells, such as how they are regulated, and how and for what applications they can be used in medical purposes.

**Hematopoietic stem and progenitor cells**

***Hematopoiesis***

Hematopoiesis, which is a combination of two Greek words: haima (blood) and poien (make/create), describes the process by which all the blood cells of an organism are generated. Through its many diverse cell types (erythrocytes, megakaryoctyes/platelets, monocytes/macrophages, granulocytes, mast cells, dendritic cells, B-cells, T-cells, and natural killer (Nk) cells), the hematopoietic system performs discrete functions including oxygen transport, removal of cellular waste and defense against pathogens. The continuity of these functions requires generation of millions of blood cells per second in an adult human[13]. The steady production of sufficient numbers of cells of all blood cell lineages, daily and throughout one's entire life, is based on a highly ordered hierarchy of the blood system (Figure 1). With their ability to self-renew and differentiate into all blood cell lineages, HSCs are the primary cells responsible for homeostatic control of hematopoiesis and therefore reside at the top of this hierarchy. The fact that a single HSC can reconstitute the entire blood system of a mouse depleted of its own hematopoietic system, highlights the importance of HSCs as the foundation of hematopoiesis[17]. Nonetheless, under normal circumstances HSCs cycle very infrequently, and a significant part of the high turnover demand is met by the highly proliferative, more committed progenitors with restricted self-renewal and differentiation capacity (Figure 1)[13].

In an adult organism, HSCs are mainly located in the bone marrow (BM) where they are supported by microenvironmental cues to retain their characteristics. The origins of BM resident HSCs, however, go back to early embryonic tissues. During mouse ontogeny, first definitive HSCs are generated in the aorta-gonads-mesonephros (AGM) region at embryonic day 10.5 (E10.5)[18–20]. Starting from E11, HSC activity is also observed in other tissues including yolk sac, placenta and fetal liver[21–23]. By E12, the number of HSCs dramatically increases in placenta and fetal liver. At around E14, the fetal liver surpasses the placenta and becomes the major HSC site until these cells colonize the thymus, spleen and finally the BM at E17. The generation of HSCs in the human embryo occurs in a similar fashion to that of the mouse, and the bulk of the HSCs reside in BM in the adulthood[24].

In the classical hematopoietic hierarchy model proposed by Weissmann laboratory[13], BM resident HSCs give rise to multipotent progenitors (MPP), which then strictly branch into oligo-potent progenitors of myeloid and lymphoid lineages (Figure 1). While the common myeloid progenitor (CMP) contain both myeloid and erythroid lineage potentials, common

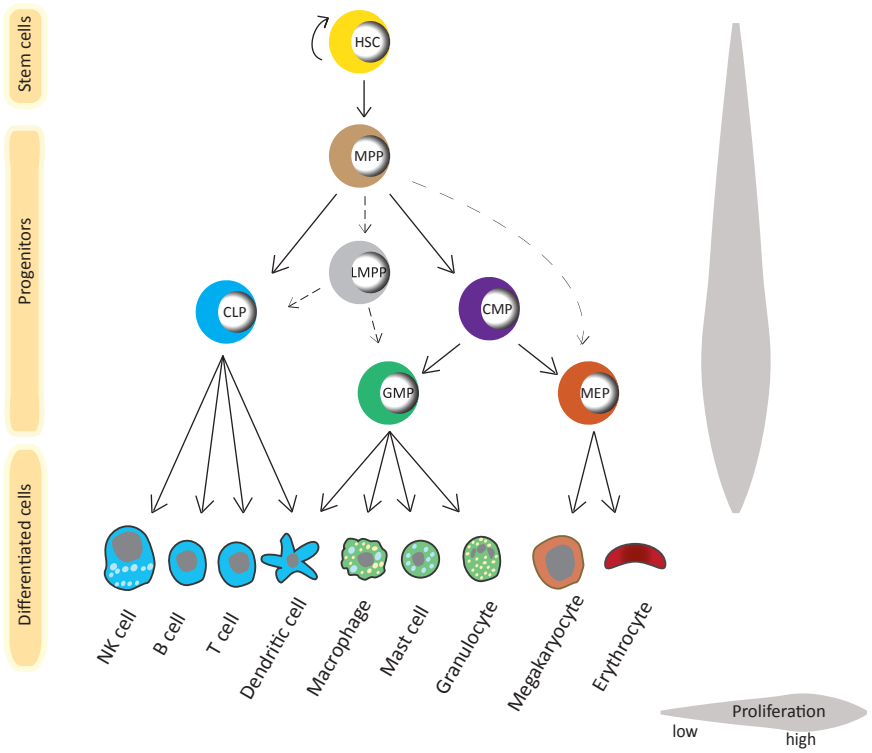


**Figure 1. Schematic overview of hematopoietic hierarchy.**
Residing at the top of the hierarchy, HSCs self-renew and give rise to a number of multipotent progenitors (MPPs), which become progressively restricted towards a specific blood lineage. The hematopoietic hierarchy model proposed by Weismann laboratory (solid arrows) suggests a strict separation of myelopoiesis and lymphopoiesis via formation of a common myeloid progenitor (CMP) and a common lymphocyte progenitor (CLP) from MPPs. The alternative model by Jacobsen laboratory (dotted arrows), however, proposes the presence of a lymphoid primed multipotent progenitor (LMPP) with both myeloid and lymphoid potential, but not the megakoryocte-erythroid potential (MEP). The progress from fairly quiescent HSCs through a variety of progenitors is generally associated with an increase in the proliferation capacity (as indicated by proliferative index), which supports the high turnover demand of blood system. HSC, hematopoietic stem cell; GMP, granulocyte-monocyte progenitor; NK, natural killer cell. (Adapted from ref[13,191]).

lymphoid progenitor (CLP) form the B-cells, T-cells and Nk cells. This model suggests that the MPPs differ from HSCs only in their self-renewal capacity and an oligo-progenitor with combined myeloid and lymphoid capacity should not exist. An alternative model proposed by Adolfsson and colleagues, however, suggests the presence of a lymphoid primed multipotent progenitor (LMPP) that maintains both myeloid and lymphoid potential, but not the megakoryocte-erythroid potential[25]. Although the classical model is strongly supported by the prospective isolation of the cell subsets it suggests (CMP and CLP), the alternative model is also compatible with the presence of these subsets. In addition, the studies showing evidence for thymic progenitors which retain myeloid potential favors the alternative model[26,27].

Owing to decades of studies, much progress in our understanding of hematopoiesis has been made. Moreover, these studies have facilitated the therapeutic success of HSC

transplantation, which is the most widely applied stem-cell therapy today. Nonetheless, the use of HSC transplantation as a treatment is still limited due to lack of compatible donors and rareness of HSCs. Therefore, much attention has been put to *ex vivo* HSC and progenitor expansion approaches. An understanding of the factors involved in regulation of hematopoietic stem and progenitor cells cell-fate decisions is, thus, required.

***Hematopoietic stem and progenitor cells identification and isolation***

Since the first demonstration of HSC functionality in the 1960s, the identification and characterization of HSCs in both mouse and man have been extensively studied. Advances in flow cytometry and transplantations into lethally irradiated mice as a functional stem-cell read-out allowed both the establishment of the cell-surface phenotype of HSCs and their prospective isolation[28]. Availability of cell-surface markers for tracking HSCs and downstream progenitors has facilitated investigations of important factors for *ex vivo* expansion or transplantation of pure populations of cells.

In the adult mouse, all functional HSCs can be found among the population of bone marrow cells that lack lineage (Lin) specific markers normally present in committed and differentiated hematopoietic cells, and express high levels of stem cell antigen-1 (Sca-1) and c-Kit. This HSC enriched mouse bone marrow cell subset is known as LSK ($Lin^-$, c-$Kit^+$, Sca-$1^+$)[29,30]. Nevertheless, only a portion of mouse LSK cells possesses the ability to reconstitute the hematopoietic system in secondary and even tertiary recipients. Since the vast majority of mouse LSK cells are multipotent progenitors, additional cell-surface markers have been identified to further subdivide this heterogeneous population. It has been shown that one-third of the LSK cells that express the signal-lymphocyte-activating molecule CD150 and are negative for CD48 and CD34 (LSK, $CD48^-$, $CD150^+$, $CD34^{low/-}$ cells) contains functional long-term reconstituting HSCs[17,28,31].

While the isolation of mouse HSCs has greatly progressed over the past decades, due to the lack of efficient *in vivo* assays, the characterization of human HSCs has been more challenging. The first cell-surface marker identified to enrich human hematopoietic stem and progenitor cells (HSPC) was CD34, which is expressed on less than 5% of all blood cells[32]. In humans, the majority of HSCs are CD34+, as evidenced by many human transplantations as well as xenograft repopulation assays, which involves the injection of human cells into immunodeficient mouse models[32]. However, CD34 also marks more lineage-restricted progenitors. Therefore, isolation of cell subsets further enriched for human HSCs also necessitates concurrent use of several cell-surface markers. Assessing long-term human hematopoiesis in non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice recipients, Bhatia and colleagues reported that human HSPC reside in the $Lin^-$ $CD34^+$ $CD38^{-/low}$ fraction[33]. Moreover, using xenotransplantations as well as complementary *in vitro* assays, Majeti and colleagues demonstrated that a $Lin^-$ $CD34^+$ $CD38^{-/low}$ $CD90^+$ $CD45RA^-$ cord blood fraction is further enriched in human HSCs[34].

***Hematopoietic stem and progenitor cells in clinics***

The start of the hematopoietic cell transplantation era coincides with the atomic era when the lethal effect of ionizing radiation was shown to be due to BM failure[35]. The rescue of the exposed recipients was possible by injection of bone marrow cells from unirradiated donors[36]. Over the last six decades, intensive research has been done on the use of HSPC

transplantations. Today, transplantation of stem and progenitor cells from BM, mobilized peripheral blood, or umbilical cord blood (UCB) is the treatment modality of choice for a variety of hematological and genetic diseases. Nevertheless, successful use of HSPC treatment is limited by a lack of human leukocyte antigen (HLA)-matched donors, high risk of graft-versus-host disease (GvHD) after transplantation and inability to expand the rare population of HSCs.

Among the different sources of HSPC for clinical applications, UCB provides some advantages over bone marrow or mobilized peripheral blood. These include the ease of collection, little or no risk to the donors, reduced risk of infection transmission, less stringent human leucocyte antigen (HLA)-matching criteria, lower risk of GvHD and rapid availability of the cord units[37]. Indeed, UCB transplantation has been shown to be beneficial in treatment of children with certain hematologic diseases and metabolic disorders[37–40]. In adult patients, however, the use of UCB as a source has resulted in delayed engraftment, a major cause of early morbidity and mortality[41,42]. This is most probably due to the low number of hematopoietic cells in a cord unit[43]. The observation that cell dose is a critical parameter for engraftment and survival, led to the initiation of studies focusing on increasing the number of cells being transplanted.

In trying to increase the cell dose in UCB transplantations, different approaches have been assessed. One of these strategies has been the use of double UCB units for transplantations[44,45]. The double cord transplantation has shown advantages over the use of single cord, including enhanced median time to achieve neutrophil recovery and lower relapse rate[43]. The growing interest in the use of double cord transplantation, however, will increase the demand for UCB units.

Another approach to reach sufficient cell numbers in UCB has been *ex vivo* expansion of HSPC[46,47]. In this approach the primary aim is to increase the numbers of HSCs present in the cord unit, which will result in permanent reconstitution of all blood lineages. However, it is as important to generate higher number of progenitors for rapid, although transient, reconstitution of the blood system, thereby decreasing early morbidity and mortality. For *ex vivo* expansion of UCB HSPC, many studies have focused on the use of particular combinations of cytokines, which are signaling molecules regulating the proliferation and differentiation of progenitors[48]. These studies suggested a requirement for the presence of cytokines which act at early stages of the hematopoietic hierarchy, including stem cell factor (SCF), Fms-related tyrosine kinase 3 ligand (Flt3L), and thrombopoietin (TPO), for expansion of HSPC[49–51]. Moreover, it has been shown that other niche factors such as stromal cells or signaling molecules, in addition to cytokines, might be essential for *ex vivo* expansion of HSCs and progenitors[52–54]. For this reason, the more we learn about the regulation mechanisms of HSPC and their microenvironment, the better we can manipulate them *ex vivo* for clinical purposes.

### *Regulation of hematopoiesis*

HSCs are functionally characterized by their ability to self-renew and their broad capacity to produce all the blood cell types. While adult HSCs mostly remain dormant during homeostasis, upon injury they dynamically regulate their numbers by cell divisions[28]. Thus, to ensure persistent blood production regardless of the situation and throughout the entire life span of an organism, the cell-fate decisions of HSCs are tightly controlled by cell-intrinsic and –extrinsic mechanisms. With regards to extrinsic factors, adult HSCs are mainly found

in specialized microenvironments of BM, referred to as niches, where they are in close proximity to supporting cells, which provide membrane bound and secreted factors[55]. A wide variety of these factors including cell-adhesion molecules, cytokines and signaling molecules, has been suggested to regulate HSC maintenance by establishing a balance between self-renewal, differentiation and survival.
Among the numerous signaling pathways and adhesion molecules constituting the HSC niche, three signaling pathways, namely Notch, Hedgehog and Wnt, have been the focus of studies over the last decade due to their putative roles in adult HSC self-renewal and differentiation[28]. Indeed, all these pathways are known to control numerous important processes during embryogenesis and adult tissue homeostasis. Nevertheless, their role in regulation of HSCs remains controversial. While, *in vitro* studies as well as mouse models with gain of function mutations suggest a positive role for these pathways, *in vivo* deletion of the key components of these pathways such as RBPjk, Smoothened and β-catenin have been shown to have no apparent effect on the HSC function[28]. Given the focus of this thesis, the early studies on the role of Wnt signaling pathway on HSC regulation will be further explained later in this chapter.

**Intestinal Stem Cells**

With a higher turnover rate than any other mammalian tissue and its unique architecture, epithelium of the intestine has been a prototype for the study of adult stem cells in health and disease. Furthermore, recent developments in the field of intestinal stem cells (ISCs) have brought many promises for therapeutic purposes. Here, I briefly explain the basic anatomy of the small intestine, its cellular composition, and ISCs.

***Basic architecture of the small intestine***

The small intestine is one of the distinct organs of the gastrointestinal tract between the stomach and the colon. The major functions of the small intestine are digestion of food and absorption of nutrients and minerals. These activities are supported by the basic structure of the small intestine consisting of three layers: an outer layer of smooth muscle responsible for rhythmic peristaltic movements that direct digested food along the intestine, a middle layer of connective tissue which harbors nerves, capillaries and lymphatic vessels mediating transport of the absorbed nutrients and an innermost layer composed of a sheet of epithelial cells called the mucosa which faces the lumen of the intestine and mediates absorption of the nutrients. To maximize the absorptive surface area, this epithelium is organized into finger-like protrusions named villi projecting into the lumen, and at the base of each villus there are multiple invaginations called crypts of Lieberkühn (Figure 2)[56].
Anatomically, the small intestine can be subdivided into three specialized parts: duodenum, jejunum and ileum (from proximal to distal). They can be distinguished based on their specific features: the presence of Brunner's glands, producing a mucus-rich alkaline secretion, in duodenum; the presence of Peyer's patches, lymphoid accumulations playing a role in mucosal immunity in ileum; and absence of both of these structures in jejunum. While the cellular composition of these subdivisions display subtle differences, they all are composed of four main types of differentiated epithelial cells (Figure 2). The absorptive enterocytes, which are characterized by luminal brush borders, are found in the villi and are the most abundant cell type throughout the intestine. Mucus-secreting Goblet cells

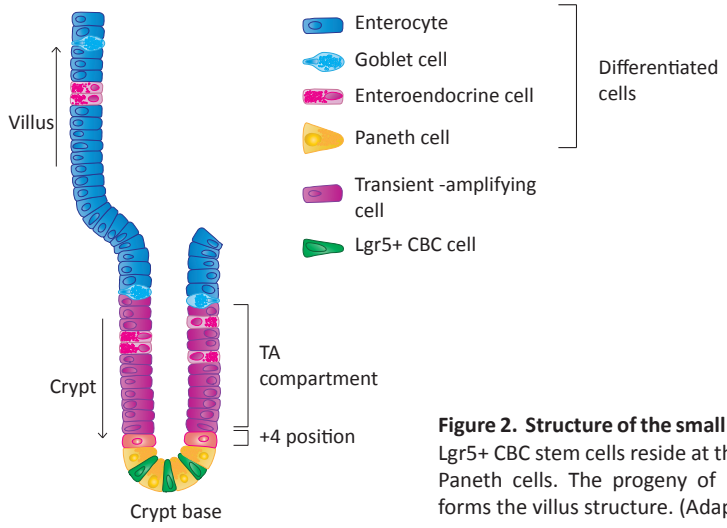


**Figure 2. Structure of the small intestine and its cell types.** Lgr5+ CBC stem cells reside at the crypt base together with Paneth cells. The progeny of Lgr5+ cells moves up and forms the villus structure. (Adapted from ref[56,192]).

are responsible for lubrication of the intestine, providing an easier passage for stool. More rare enteroedocrine cells secrete hormones aiding in the control of the gut functions. Both goblet and enteroendocrine cells can be found in the villi and crypts. Finally, Paneth cells, which reside exclusively at the bottom of the crypts, secrete bactericidals such as lysozymes and defensins.

### *Epithelial renewal in the intestine*

Fulfilling its activities, the small intestine is exposed to considerable environmental insults, which impose a requirement for continuous renewal of the epithelial lining of its lumen. Indeed, the simple, columnar epithelium lining the villi and the crypts is completely renewed every 3-5 days in mammals[57]. Therefore, this extreme turnover rate necessitates an efficient self-renewal mechanism to maintain the intestinal tissue homeostasis. While the epithelia covering the villus are post-mitotic, the crypts harbor vigorously proliferating epithelial cells and therefore form the engines of this dynamic renewal process. Each crypt produces about 250 new epithelial cells every day and up to ten crypts supplies cells to a single villus.
Residing at the bottom of the crypts, intestinal stem cells (ISCs) are the driving force behind the dynamic turnover of intestinal epithelium (Figure 2). The ISCs generate rapidly expanding progenitors known as transit-amplifying (TA) cells, which take about twelve hours for each of their four to five cell cycles. As they proliferate and move upward, TA cells start to differentiate into enterocytes, Goblet or enteroendocrine cells and exit the crypt onto the villus. These differentiated, functional epithelial cells continue migrating along this conveyor belt until they reach the tip of the villus where they undergo apoptosis and are shed into the lumen of the intestine. The much longer-lived Paneth cells, which are the only differentiated cell type, escape the upward migration and reside in the crypt base where they live 6-8 weeks[58].

***Adult stem cells of the intestine***

Early studies towards identifying the intestinal stem cells led to two alternative stem cell models[59]: the crypt base columnar cell (CBC) model and the +4 cell model, respectively.
In the early 1970s, Cheng and Leblond discovered the presence of slender, immature cycling cells, known as crypt base columnar (CBC) cells, interspersed among the Paneth cells[60]. Moreover, they performed a rudimentary lineage tracing study by radiolabeling strategy which supported the notion that CBC cells might be the multipotent cells of the intestine[61]. Later on, by using a genetic marking method induced by chemical mutagens, Winton and Ponder demonstrated the clonal nature of crypts as well as the flow of cells from crypts to villus tips as 'ribbons' for the first time[62]. By use of a similar marking strategy, Bjerknes and Cheng showed the presence of both long-lived and short-lived crypt cells corresponding to a stem cell and TA cells compartment, respectively[63]. These long-lived clones consistently included a CBC cell and gave rise to all four differentiated types of epithelial cells of the intestine, providing additional support for the model of CBC cells as intestinal stem cells. With these observations, Bjerknes and Cheng further developed their stem cell zone hypothesis[58,63,64], and stated that CBC stem cells reside at the bottom of the crypts with Paneth cells and only exiting the stem cell zone around the +5 position (one cell diameter distance from the uppermost Paneth cell) lead to their differentiation.
The alternative of the CBC cell model, referred to as 'position 4' or '+4' model was proposed based on the prediction of a common cell origin at position 4-5 just above the Paneth cell compartment (Figure 2)[65]. Later, Potten and colleagues reported the presence of radiation sensitive, DNA-label retaining cells residing directly above Paneth cells at the +4 position[66,67]. The radiation sensitivity of these cells was considered to be a beneficial stem cell characteristic for prevention of accumulating carcinogenic genetic changes. Moreover, label-retention is usually taken as indicative of mitotic quiescence, however in this case +4 cells were cycling every day. This label-retaining trait was instead proposed to be a consequence of asymmetric segregation of the labeled and unlabeled DNA into the stem cells and their progeny, respectively[67]. Indeed, the 'immortal strand' theory[68] suggests asymmetric segregation of chromosomes as a protection from accumulating mutations. However, this hypothesis has not been proven since. On the contrary, stem cells cycling at the intestinal crypts randomly segregate their chromosomes[69,70].

***Lgr5+ stem cells and organoids***

Since the proposal of the intestinal stem cell models, many studies have focused on identification of stem cell markers, which would not only enhance the understanding of small intestine biology but also potentiate the isolation and exploitation of these stem cells for regenerative purposes. However, for a long time the ISC field had been challenged due to the fact that most of these studies were solely based on positional information of potential markers expression, and they were not supported by direct evidence for stemness as assessed by lineage tracing or transplantations[59]. While the evidence for the +4 cell model is still confusing and controversial, in 2007 Barker and colleagues have identified an exquisite CBC cell marker, Lgr5, confirming Leblond's predictions regarding CBC cells.
The road to the discovery of Lgr5 as stem cell marker was paved with accumulating knowledge about the role of deregulated Wnt signaling in colon cancer and physiological Wnt activation in crypt proliferation. The analysis of Wnt target genes in human colon cancer cell lines

as well as intestinal crypts revealed a number of genes[71,72] among which Lgr5 turned out to be uniquely expressed by the CBC cells in the crypts of the small intestine[12]. Lineage tracing studies with an inducible Lgr5 reporter mouse model showed the formation of clonal ribbons, which persisted throughout the life and contained all different cell types of the intestine. These further demonstrated the longevity and multipotency of CBC cells.

In contrast to many other adult stem cell types, Lgr5+ CBC stem cells of the intestines divide every day. This corresponds to around 1000 cell cycles in a laboratory mouse lifetime, thus defies the Hayflick phenomenon which states that normal human cells have limited capacity to divide[59]. However, until the work of Sato and colleagues, it was not possible to culture these highly proliferating stem cells. In his work, Sato carefully analyzed the growth factor requirements of the mouse intestinal crypts by taking into consideration the genetic evidence of stem cells self-renewal, differentiation and carcinogenesis. Consequently, he discovered that a combination of three growth factors, namely epidermal growth factor (EGF), Noggin (a bone morphogenic protein (BMP) signaling inhibitor) and R-spondin (a Wnt signaling enhancer), were essential for culture of ISCs *in vitro*[73]. Culturing mouse intestinal crypts or single Lgr5+ CBC cells also required a basement membrane extract, also known as Matrigel, as a scaffold for three-dimensional (3-D) growth. In this culture system having the proper signals and conditions, Lgr5+ cells formed ever-expanding 'organoids', mimicking the crypt-villus structure of intestines (Figure 3). These organoids, also known as 'mini-guts' occur as cysts composed of a central lumen and surrounding crypt-like budding structures (Figure 3). While post-mitotic differentiated cells migrate towards the central area facing the lumen, Paneth and Lgr5+ stem cells reside together in these buds. With their self-renewal kinetics, longevity and capacity to generate all the cell types of the tissue, the organoids form an *in vitro* equivalent of mouse small intestinal tissue.

The functional readout proving the true value of organoid system as a physiological model came from studies done with mouse colon organoids. Organoids derived from a single Lgr5+ stem cell of mouse colon has proven to be transplantable into multiple recipient mice with chemically induced epithelial damage. The grafted organoids formed patches lasting at least six months without any apparent histological changes[74]. Thus, the invention of the groundbreaking organoid culture system further demonstrated the stemness of Lgr5+ stem cells in the intestines.

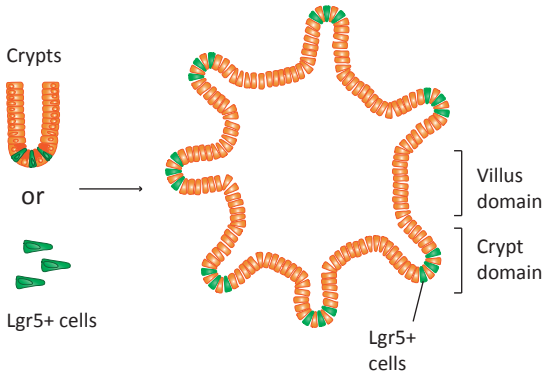


**Figure 3. Schematic representation of a small intestinal organoid.** Organoids generated from either crypts or single Lgr5+ stem cells consists of a central lumen lined by villus- and crypt-like domains. (Adapted from ref[73]).

The establishment of organoid culture system not only provided a system to study intestinal biology but also offered a great tool for disease modeling and therapeutic purposes. For example, human intestinal organoids were successfully applied as a disease model for cystic fibrosis[16]. While the wild-type organoids swell upon cAMP treatment due to the lack of cystic fibrosis transmembrane conductor receptors (CFTR), organoids derived from cystic fibrosis

patients do not respond to this treatment. Furthermore, after restoring the CFTR locus via genome-editing tools, the diseased organoids regained their cAMP responsiveness. In addition, human intestinal organoids could be engineered to mimic the *in vivo* situations of colon cancer[75] and the organoids derived from cancerous tissues can reflect their genetic regulation and be used for *in vitro* drug testing for clinical treatment predictions[15]. Collectively, the establishment of human intestinal organoids not only provides a valuable tool for basic research but also offers many novel strategies for translational medicine.

**Liver stem cells**

***Liver***

The liver is a central organ for maintenance of systemic homeostasis of an organism. It performs a wide range of functions including nutrient metabolism, drug detoxification, glycogen storage, production of serum proteins and bile secretion. The basic architectural unit responsible for these functions of the liver is called a hepatic lobule (Figure 4)[76]. At each corner of this polygonal lobule, there is a portal triad of vessels consisting of a portal vein, hepatic artery and bile duct. Blood from the portal vein and hepatic artery flows towards a central efferent vein through sinusoids lined with fenestrated sinusoidal endothelial cells, which facilitates the exchange of materials between blood and hepatocytes. Most of the functions of the liver are carried out by hepatocytes, which are the main parenchymal cell type of the liver. These highly polarized epithelial cells comprise approximately 80% of total liver volume and are arranged in cords. Tight junctions between the hepatocytes form bile canaliculi, which are linked to bile ducts. Bile ducts are composed of the other parenchymal cell type of the liver known as biliary epithelial cells or cholangiocytes. Bile salts produced in mature hepatocytes are excreted through bile canaliculi and bile ducts into the duodenum of the small intestine. Kupffer cells and hepatic stellate cells (Ito cells), which together with fenestrated endothelial cells reside in sinusoids, are the other non-parenchymal cells of the liver. While Kupffer cells, resident macrophages of the liver, are located at the luminal side of sinusoids, hepatic stellate cells, playing a role in extracellular matrix remodeling upon damage and secretion of a variety of growth factors, reside at the opposite side.
As the liver filters the blood it receives from the portal vein, it is inherently exposed to harmful compounds. Moreover, it can be subjected to insults from a variety of hepatotropic viruses, leading to liver injury. Due to its vital roles, however, loss of liver function is often fatal. To compensate for any injury-induced loss of liver mass and to continue to exert its functions, the liver displays a remarkable regenerative capacity[77]. It is therefore also possible to transplant liver tissue from a living donor.

***Liver regeneration***

In an adult liver under physiological conditions both hepatocytes and cholangiocytes are infrequently dividing[76]. The normal turnover of mature hepatocytes occurs slowly over more than several months[78]. Following injury-induced loss of liver mass, however, an adult liver displays great regenerative response. When two-thirds of a rat liver is removed by partial hepatectomy (Phx), the remaining hepatocytes and ductal cells from the undamaged lobes re-enter the cell cycle and replace the lost hepatic mass within 5-7 days (8-15 days in humans)[77]. The regenerative capacity of mature hepatocytes has further been demonstrated by serial

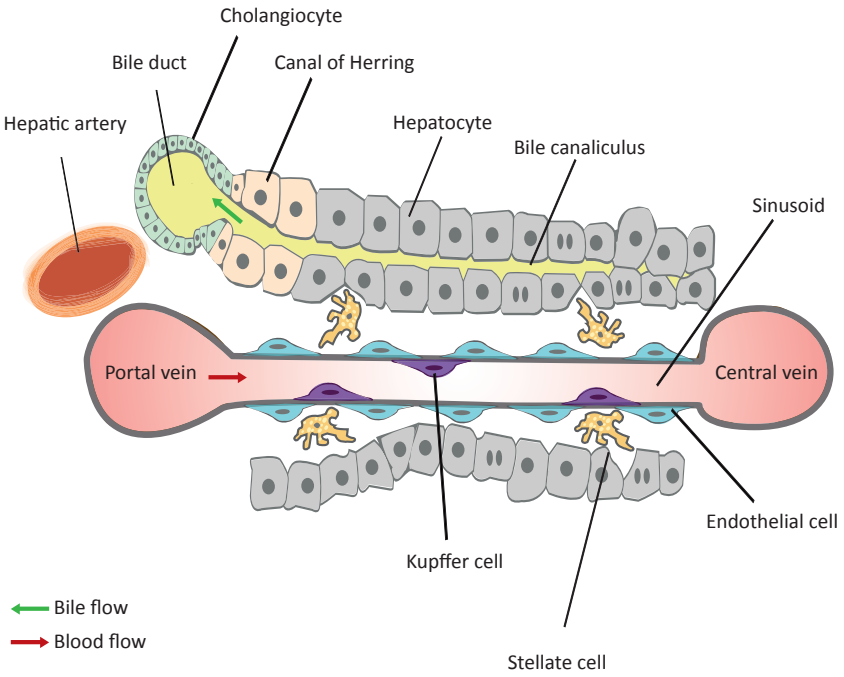


**Figure 4. Schematic overview of hepatic lobule.**
In the liver lobule, the portal vein, hepatic artery, and bile ducts together comprise the portal triad. Mixed blood from the portal vein and the hepatic artery proceeds through the sinusoids surrounded by fenestrated hepatic sinusoidal endothelial cells towards the central vein. Bile produced by the hepatocytes is collected via bile canaliculus and flows in the opposite direction towards the bile duct. While liver resident macrophages, Kupffer cells, are located at the lumen of sinusoids, stellate cells reside in the space between hepatocytes and sinusoids. The canal of Herring forms the junction between hepatocytes and bile duct. (Adapted from ref[76]).

transplantations upon which a small number of normal hepatocytes reconstituted diseased livers multiple times, dividing at least 80 times in the process[79]. However, in situations where the proliferation capacity of hepatocytes is compromised, another mode of regeneration process mediated by a population of liver stem/progenitor cells (LSPCs) is proposed to take over the responsibility of differentiation towards hepatocytes[76]. The concept of LSPCs has originated from studies in rats where the combination of hepatocyte proliferation blockage by DNA damage and Phx gave rise to a population of cells with oval nuclei, thus named as oval cells, from the biliary tree[80]. Rat oval cells are shown to express both hepatocyte and cholangiocyte markers, and appear to possess bipotential capacity to differentiate into both of these cell types[81]. The term oval cell has since been used as an anonym to LSPCs, at least in rats. However, the oval cell bipotentiality was not clearly demonstrated in rats. As a result of the lack of genetic tools in rats and a specific marker to identify oval cells, the mouse has become the organism of choice for LSPC studies. Soon, it was realized that the injury regimens leading to oval cell response in rats did not result in the same effect in mice. Therefore, for oval cell induction in mice, alternative injury models mediated by hepatotoxins (e.g., 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-containing diet[82] or choline-deficient ethionine-supplemented (CDE) diet[83]) have been devised. Despite the emergence of mouse oval cells, the injuries sustained in these models differ from that of the

rat model. It has also been shown that mouse oval cells, which constitute a heterogeneous population, display differences compared to their rat and human counterparts[84]. For example, unlike in rat and human, mouse oval cells do not express the immature hepatocyte marker, alpha-fetoprotein[85]. Despite different characteristics and etiology of LSPCs, several common markers in mice, rats and humans, including CK19, EpCAM, and CD133, have been identified to describe these cells[86–89]. Subsequent isolation of LSPCs based on these markers and their *in vitro* culture have demonstrated that LSPCs contain cells that are clonogenic and can differentiate into both hepatocytes and cholangiocytes[90]. Interestingly, cholangiocytes isolated from normal livers based on the same markers also display the same bipotential capacity *in vitro*, raising the question whether they constitute a reserve stem cell population that can give rise to LSPCs upon damage[76]. In support of this notion, several lineage-tracing studies done with cholangiocyte-specific Cre driver strains collectively indicated that LSPCs emerging upon certain injury conditions are indeed derived from cholangiocytes[91–93]. However, a variety of more recent studies strongly argue against the presence of a stem cell population as a genuine source of hepatocyte formation in liver[94–97]. Nevertheless, given the capacity of hepatocytes to regenerate even in severe injury models, the identification of a potential stem cell population becomes rather difficult. Adding up to the complexity of the identification of liver regenerating populations, it has been suggested that if regenerative capacity of one of the epithelial compartments of the liver is compromised, the other one can give rise to the proliferation-defective compartment[81].

***Lgr5+ liver stem/progenitor cells and organoids***

Despite the complications of *in vivo* identification of stem cell populations, several groups isolated liver stem cell populations from the adult liver based on marker gene expression. An important example for this comes from the studies of Huch and colleagues who demonstrated the appearance of Lgr5+ LSPCs upon acute liver damage with carbon-tetrachloride ($CCl_4$) as well as upon an oval cell response by DCC or CDE diets [11]. Lgr5+ LSPCs are shown to actively contribute to liver regeneration by giving rise to both hepatocytes and cholangiocytes *in vivo*. More importantly, these cells can be cultured as organoids for over a year, while they maintain their bipotency to differentiate into hepatocytes as well as bile duct epithelial cells. While the origin of Lgr5+ LSPCs remains to be determined, liver organoid cultures can also be initiated with bile ducts instead of Lgr5+ cells. Owing to their capacity to expand indefinitely and differentiate into hepatocytes, organoids also repopulate livers of mice with congenital metabolic liver disease and contributed to restoration of their hepatic functions[14]. With this study, a robust culture of mouse LSPCs has been established for the first time. Furthermore, recently Huch and colleagues also established long-term culture of genomically stable human organoids from adult human liver[98]. As in the case of intestinal organoids, the liver organoid technology opens up new avenues for disease modeling, drug testing, gene therapy and transplantations[99].

**Signal transduction pathways in regulation of self-renewal and differentiation of adult stem cells**

A central question in applying stem cell technologies to clinics is how the self-renewal and differentiation of stem cells are controlled. In order to increase the number of stem cells or guide them towards a specific lineage, it is of vital importance to know how to

manipulate these cells in a predictable and reproducible manner. Over the years, many microenvironmental factors that regulate stem cell fate decisions have been discovered. Among these external cues including members of BMP, Hedgehog, Wnt and Notch families, Wnt proteins stand out due to their roles in a variety of stem cells types[100]. Therefore, in the next part of this introduction, I will give an overview of the Wnt signaling pathway, Wnt proteins and their roles in regulation of adult intestinal, hematopoietic and liver stem cells in culture.

**WNT SIGNALING**

With the identification of the segment polarity gene *Wingless (wg)* as the fly homologue of the mouse proto-oncogene Integrase-1 (Int-1), a new termed was coined for Int-1 and related genes: Wnt1 and the Wnt gene family, respectively[101]. Ever since, the Wnt genes and encoded proteins have been widely studied in a variety of different organisms such as worms, flies, mice and humans and in many different contexts including development, tumor formation, and stem cell regulation[102,103]. Owing to these studies, our understanding of the wide range of processes Wnt ligands control as well as how Wnt proteins are produced, are secreted and signal has greatly expanded.

Signaling through the Wnt family of secreted glycoproteins is an evolutionarily conserved mechanism in all metazoans[104]. In mammalians, Wnt ligands play key roles in numerous processes including cell proliferation, cell fate determination, differentiation and survival during embryogenesis and adult tissue homeostasis[102,103]. With respect to maintenance of adult tissue homeostasis, the effects of Wnt signals in regulation of stem-cell fates from a variety of different tissues including intestines, hair follicles and hematopoietic system, are particularly important[105].

Activation of Wnt signaling cascades proceeds in a series of events including the production and secretion of active Wnts, binding to transmembrane receptors, activation of cytoplasmic effectors, and transcriptional regulation of gene expression. Wnt proteins can relay their signals through at least three different pathways, including the 'canonical' β-catenin pathway, and the 'noncanonical' planar cell polarity and $Ca^{2+}$ pathways[106]. The Wnt-β-catenin pathway is so far the most extensively studied and best-understood Wnt signaling cascade (Figure 5). In the absence of a Wnt protein, cytoplasmic β-catenin is sequestered by a destruction complex, which is composed of the scaffolding protein Axin, adenomatous polyposis coli (APC), casein kinase 1 (CKI) and glycogen synthase kinase 3β (GSK-3β)[107]. The kinases of the destruction complex, CKI and GSK3β, sequentially phosphorylate β-catenin, which is then recognized by a ubiquitin E3 ligase subunit, known as β-Trcp, and targeted for proteosomal degradation. Due to its elimination, cytoplasmic β-catenin cannot reach the nucleus, where Wnt target genes are repressed by the DNA binding T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins[108]. Upon binding of a Wnt protein to the N-terminal cysteine-rich domain (CRD) of Frizzled (Fz) family of receptors and to low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6 co-receptors, Axin is recruited to the receptor complex at the cell membrane. The resulting inhibition of the destruction complex leads to stabilization of cytoplasmic β-catenin, which accumulates and translocates into the nucleus where it forms complexes with TCF/LEF transcription factors to activate Wnt target gene expression[104].

As many studies indicated roles for Wnt-β-catenin pathway in stem cell self-renewal and differentiation in diverse tissues *in vivo*[103], the manipulation of this pathway, in particular by

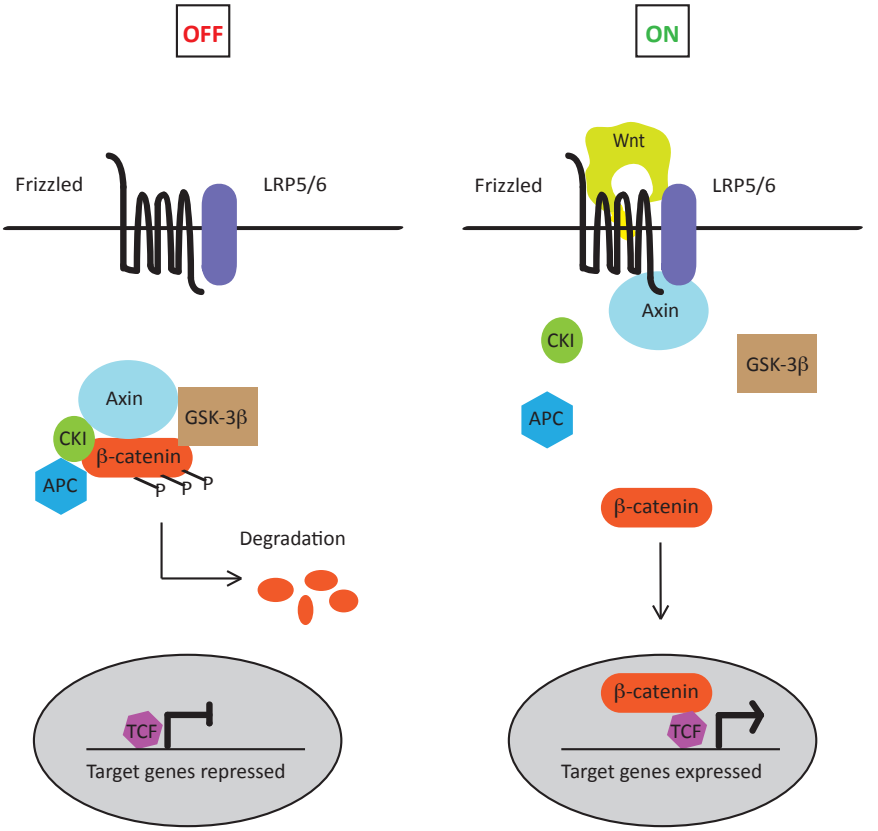


**Figure 5. The canonical Wnt signaling pathway.** In the absence of a Wnt ligand (OFF state), the destruction complex, composed of a scaffolding protein Axin, adenomatous polyposis coli (APC), casein kinase 1 (CKI) and glycogen synthase kinase 3β (GSK-3β), phosphorylates β-catenin. Phosphorylated β-catenin is targeted for degradation by the proteasome. Therefore cytoplasmic β-catenin remains low and Wnt target genes are repressed. Binding of a Wnt ligand to its cell surface receptors Frizzled (Fz) and co-receptors low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6 (ON state) leads to inhibition of the destruction complex. In turn, stabilized β-catenin translocate into the nucleus where it interacts with TCF/LEF transcription factors to regulate transcription of Wnt target genes. (Adapted from ref[102]).

Wnt proteins have proved to be important for the study of stem cells *in vitro* both for basic research and for therapeutic purposes[15,98,109–111]. Therefore, it is of great value to understand the biochemistry of Wnt proteins in terms of achieving stably active Wnt proteins for *in vitro* studies of stem cells.

**Wnt proteins: production and secretion**

In mammalian genomes, including the human genome, there are 19 Wnt genes, which encode for cysteine-rich proteins of approximately 350-400 amino acids. These proteins bear an N-terminal signal peptide, that targets them to the endoplasmic reticulum (ER)[104]. Although Wnt proteins were discovered more than three decades ago and are secreted, difficulties in efficient purification of Wnts have precluded their thorough biochemical

characterization and use for *in vitro* studies. Whereas early studies predicted a highly soluble nature for Wnt proteins which contain several highly charged amino-acids and many potential glycosylation sites, initial attempts to purify the founding Wnt protein, Wnt1, has revealed its poor solubility and high association with the cell surface and extracellular matrix[112]. Nevertheless, some other Wnts such as Wg and mouse Wnt3a have proved to be relatively more soluble[113,114]. Thus, biologically active Wg and Wnt3a could be harvested in the form of conditioned-media from the specific cell-lines overexpressing them. The cell-free, serum-containing conditioned-medium of mouse Wnt3a has been extensively used in many studies, despite the many undefined factors it harbors that may complicate the analysis of any biological assay. With respect to obtaining a more defined system, the purification of biologically active mouse Wnt3a for the first time has been a major step for the Wnt field[115]. Moreover, this same study led to the discovery that Wnt proteins are lipid-modified, and provided insights into the hydrophobicity and insoluble nature of the Wnts.
During their journey from the ER to the extracellular environment, Wnt proteins (except *Drosophila WntD*[116]) associate with several enzymes and undergo several post-translational modifications, including N-glycosylations and lipidations (Figure 6A). The numbers of glycosylations appear to differ among Wnt proteins. For example, while mouse Wnt1 carries up to four glycosylation sites[117], mouse Wnt3a is glycosylated at two sites[118]. Moreover, while mutations in the glycosylations sites of mouse Wnt1 seem to have only minor effects on protein activity, glycosylations of Wnt3a protein have been suggested to be necessary for their proper folding and lipid modifications[118]. While the exact functions of glycosylations in different Wnts is still under debate[112,117,118], it is now clear that the addition of lipid-moieties, in particular the addition of a mono-saturated fatty acid, known as palmitoleic acid, to a conserved serine residue in Wnt proteins is absolutely essential for their activity and may be crucial for their secretion[103,112]. The addition of palmitoleic acid to Wnt proteins is thought to be mediated by an ER resident multipass transmembrane protein, called Porcupine (PORCN), which contains an O-acyl transferase domain (Figure 6A)[112,119]. This is evidenced by the observations that both mutating the conserved serine residue (S209 in mouse Wnt3a) to be lipid modified and loss of PORCN results in the accumulation of the mutant Wnt protein in the ER[119].
Release of Wnt proteins from the secretory machinery requires the action of two additional proteins: Wntless (WLS) (also known as Evenness interrupted (EVI) or Sprinter (SRT)) and the retromer complex (Figure 6A)[112]. WLS is a multipass transmembrane protein localized to the ER, Golgi, the plasma membrane and the endosomes between them, and is essential for Wnt secretion[120–123]. WLS functions as a sorting receptor by binding and accompanying Wnts to the cell surface. The binding of WLS to Wnts takes place only when Wnts are lipid-modified, indicating that WLS acts downstream of PORCN[124]. Once WLS escorts a lipid-modified Wnt protein to the plasma membrane, it is recycled back from endosomes to the Golgi by the actions of retromer complex and from the Golgi to the ER by specific vesicles for multiple rounds of Wnt secretion[123,125–129]. A deficiency of retromer complex function results in degradation of WLS in the lysosomes and a strong Wnt secretion defect.

**Solubility and purification of Wnt proteins**

The lipid modifications of Wnts are necessary for their intracellular trafficking as well as for their activity upon secretion. Structural analysis of the Xenopus Wnt8 protein as bound to Fz has shown that specifically palmitoleic acid lipids are involved in engagement with

the Fz receptors[130]. However, the questions of how such hydrophobic molecules, which tend to tightly associate with membranes, move in the extracellular environment and are conveyed to their targets remains elusive. Several mechanisms have been proposed that may act together to mediate Wnt solubility in the extracellular environment (Figure 6B). One mechanism facilitating Wnt proteins presentation throughout the extracellular matrix employs heparan sulfate proteoglycans (HSPGs) consisting of a core protein to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached[112]. It has been suggested that by attaching to the GAG chains of HSPGs at the cell surface, Wnt proteins retain their solubility and might move along the extracellular matrix, creating gradients in a restricted diffusion manner (Figure 6B)[131]. Nevertheless, their role in diffusion of Wnts remains unclear, as most recent findings argue against long-range spreading of Wnts[132].

Although plasma membrane tethering of Wnt proteins is a feasible model for their activity, the fact that biologically active Wnt proteins (e.g. mouse Wnt3a and Wnt5a) can be obtained from conditioned-media and upon further purification suggests that Wnt proteins might use alternative carriers. Indeed, several *in vivo* carriers such as lipoprotein particles or exosomes have been suggested to incorporate Wnt proteins, shielding their lipids from the aqueous environment and enabling Wnts to travel in the extracellular space between the cells (Figure 6B)[133–136]. Worthwhile to note is that lipoproteins have been shown to interact with HSPGs,

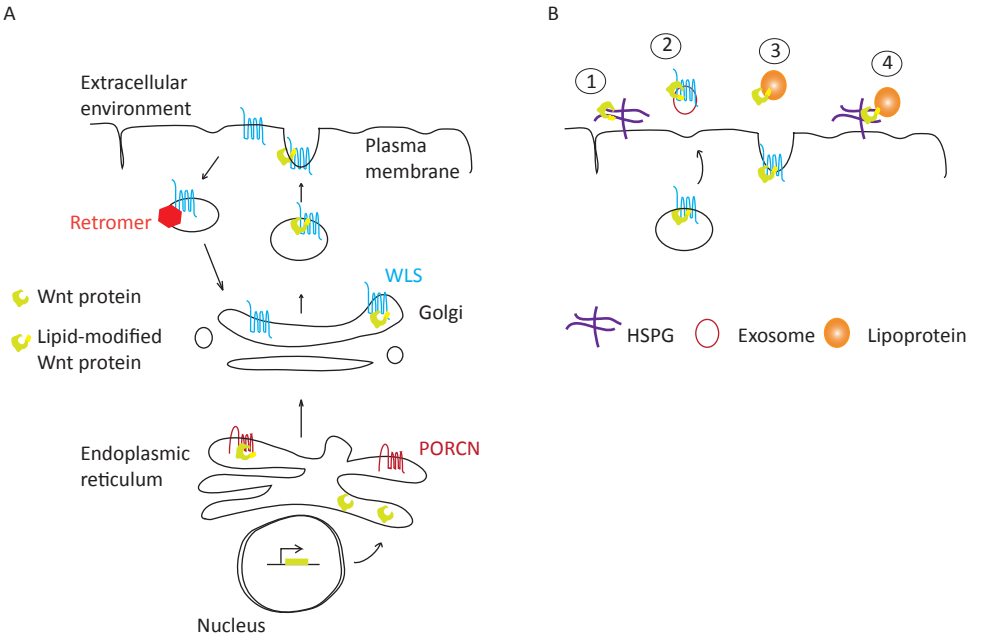


**Figure 6. Model of Wnt secretion.**
(A) After their translation, Wnt proteins are lipid-modified by Porcupine (PORCN) in the endoplasmic reticulum (ER), where newly lipidated Wnts also bind to their sorting receptors, Wntless (WLS). WLS escort Wnt proteins from the ER to the plasma membrane. Efficient Wnt secretion necessitates recycling of WLS from the plasma membrane back to the ER. Retromer complex dependent endosome to Golgi trafficking brings WLS to the Golgi, while retrograde movement from the Golgi to the ER is mediated by specific vesicles. (B) Wnt proteins release and solubility at the extracellular environment includes several possible mechanisms: Wnts might associate with the heparan sulfate proteoglycans (HSPGs) (1), exosomes (2), lipoprotein particles (3), or HSPGs and lipoprotein particles together. (Adapted from ref[112,123,193,194]).

suggesting that these carriers can work together in Wnt distribution in the extracellular environment (Figure 6B)[137].
During purification of Wnt proteins, *in vivo* carriers that maintain Wnt in an active confirmation may be lost and might be partially replaced by CHAPS detergent micelles[138]. Upon dilution in cell-culture media, these micelles are dissolved and purified Wnt proteins tend to aggregate unless stabilized by serum[115,138–140]. It has been demonstrated that the addition of HSPGs could maintain the solubility and activity of purified Wnt3a in the absence of serum[138]. Besides this, association with liposomes, which are spherical nanovesicles composed of phospholipid layer(s), also promote the solubility and activity of purified Wnt3a protein[141,142].
Given the importance of Wnt signals for a number of cellular processes including regulation of stem-cell fate *in vitro*, the engineering of purified Wnt molecules with hydrophobic carriers such as liposomes, to achieve high level of stable Wnt activity in serum-free culture conditions, bears significant potential for practical use.

**Wnt signaling in regulation of HSPC**

With the realization that many Wnt proteins are expressed in blood cells of both mouse and human, and the Wnt signaling pathway is deregulated in several hematological malignancies, much interest has been directed to a possible role for Wnt signaling in the control of self-renewal of HSC and proliferation of progenitors[143–146]. However, varied experimental approaches resulted in conflicting outcomes leading to a controversy in the field and making the role of Wnt signaling in HSPC difficult to generalize.
Following their observation on the activation of a Wnt-β-catenin reporter in mouse HSPC, Reya and Willert demonstrated that stimulation of Wnt signaling by either a constitutively active form of β-catenin or by purified Wnt3a protein induced expansion of apoptosis-resistant, transgenic mouse HSPC *ex vivo*[115,147]. The importance of Wnts in hematopoiesis has been heightened by studies showing defective fetal liver and bone marrow HSC self-renewal in mouse models carrying a null allele of Wnt3a or overexpressing the Wnt receptor antagonist Dickkopf1[148,149]. However, using mouse models in which continuous Wnt activation was achieved via inducible stabilization of β-catenin, two other studies reported multilineage differentiation block and exhaustion of HSCs[150,151]. Together, these studies suggest that the context and/or duration of Wnt activation might be important determinants of the physiological outcome, and other factors in conjunction with Wnt signals might play a role in maintenance/expansion of HSPC.
Given the importance of HSPC for therapeutic purposes, several studies have investigated the potential use of Wnt signals for *ex vivo* expansion of normal HSPC. In this regard, Nemeth and colleagues analysed the impact of both mouse Wnt3a and Wnt5a proteins in *ex vivo* culture of HSPC and reported that while Wnt3a protein induce a decrease in the number of HSPC, Wnt5a improve repopulation capacity of mouse HSPC[152]. However, in their study Oziemlak and colleagues showed an opposite effect of purified Wnt5a, which was shown to obstruct the repopulation capacity of mouse HSPC[153]. The different outcomes of these studies can be attributed to culture systems with varying parameters, including the amount and the type of cytokines used and level and duration of Wnt activity. However, the question of whether Wnt signals could inhibit differentiation and induce proliferation of HSPC *ex vivo*, in mice and particularly in humans, remains unanswered.
The maintenance of homeostasis in the hematopoietic system is achieved via balancing

the proliferation, apoptosis and differentiation of HSPC. This intricate tuning underlies the difficulty in assigning the role of Wnt signals in complex systems or in systems where several factors are missing. With respect to this, a line of studies suggested that active β−catenin increases apoptosis of HSPC, which might require survival signals for maintenance/expansion[154,155]. Moreover, it has been reported that the activation of Wnt pathway in recipient mice via a GSK3β inhibitor, which activates other cascades in addition to Wnt, increases the numbers of HSPC and repopulation *in vivo*[156]. However, the study of Luis et al. suggested that activation of Wnt signals at a low level enhances the repopulation capacity of HSPC, regardless of activation of another pathway. Taken together, these studies suggest a role for Wnt signaling in HSPC maintenance/expansion given it is delivered in the right way and/or with the adequate accessory signals.

**Wnt signaling in ISCs and organoids**

The early genetic analyses of intestinal crypt physiology revealed that the dominant force behind the crypt proliferation in the intestine is canonical Wnt signaling. Inhibition of Wnt activity by deletion of Wnt effector TCF4 was reported to completely block the development of intestinal crypts in neonatal mice[157]. Expression of the Wnt inhibitor Dickkopf1[158] in adult mice resulted in loss of crypts. The same phenotype was also observed upon conditional deletions of β-catenin[159] or TCF4[160] underlying the Wnt-dependence of adult crypt homeostasis. Given the importance of Wnt signaling in crypt proliferation, it is not surprising that aberrant activation of the Wnt pathway is frequently observed in colon cancers. Comparative analysis of colon cancer lines and normal colonic epithelium revealed that most Wnt regulated genes are expressed throughout the crypt[71], fitting the role of Wnts in regulation of stem and TA cell proliferation[157], as well as Paneth cell differentiation[161]. Further investigation of a small set of Wnt regulated genes whose expression was restricted to the crypt base[72], led to the discovery of Lgr5 as an exquisite ISC marker[12].

The essential role of Wnt signaling in intestine biology has also been reflected *in vitro* with the establishment of small intestinal organoids which require addition of Wnt signaling agonists, R-spondins[73]. R-spondins were later on shown to be the ligands for Lgr5 proteins[162–164]. By binding to these receptors, R-spondins stabilize the Wnt-Fz-Lrp receptor complex, enhancing the duration and strength of Wnt activity[165]. This also suggested a requirement for Wnt producing cells for small intestinal organoid cultures. In this regard, the formation of self-organizing mini-guts from single Lgr5+ stem cells in the absence of a non-epithelial niche was both surprising, and suggestive of the presence of epithelial niche cells. Adjacent to the Lgr5+ ISCs both *in vivo* and *in vitro*, Paneth cells were hypothesized to constitute such niche cells. Indeed, the efficiency of organoid formation dramatically increased for isolated stem cell/Paneth cell doublets in comparison to single Lgr5+ stem cells, which very inefficiently survived[166]. Microarray analysis of Paneth cells revealed the expression of important growth factors, such as Wnt3, EGF and Notch ligands, remarkably similar to the mini-gut culture system. Supporting the notion of Paneth cells as niche components, Wnt3 mutant Paneth cells fail to support organoid growth, which could be rescued by addition of exogenous Wnt[167].

Another example for the importance of proper signals or niche components for *in vitro* culture of stem cells came from the mouse colon. The normal mouse colon epithelium which also bears Lgr5+ stem cells[12], lacks the Paneth cells and produces insufficient amounts, if any, of secreted Wnt ligands for colon crypt culture[168]. In line with this, the growth of mouse

colon organoids requires an additional Wnt source[109]. Similar to the mouse colon example, the culture of organoids from human tissue samples was initially not successful. Screening a number of different growth factors, Sato and colleagues discovered the necessity for additional factors among which exogenous Wnt takes the first place. By addition of a Wnt3a conditioned-medium, it became possible to culture human small intestine and colon tissues long-term[109,169].

**Wnt signaling in liver stem/progenitor cells and organoids**

Whereas the factors that maintain stem cells in the intestine are extensively studied, the niche that provides essential cues for facultative liver stem/ progenitor cells is less well defined. Nonetheless, the injury-induced appearance of Lgr5+ LSPCs in liver has indicated that Wnt signaling constitute a core signaling pathway involved in stimulation of a regenerative response[11].
Upon chronic liver damage, myofibroblasts and macrophages have been shown to surround the emerging progenitor cells[170]. The secretion of Wnt ligands, in particular Wnt3a and Wnt7a, by macrophages, has been shown to promote hepatocyte regeneration from LSPCs. The importance of Wnts in emergence of liver progenitors has further been highlighted by studies demonstrating a role of β-catenin for the oval cell response[171,172]. While inhibition of Wnt signaling via conditional deletion of β-catenin dramatically decreased the number of emerging progenitors[171], a constitutively active form of β-catenin expands the pool of these cells[172]. Similar to Wnt proteins, R-spondin levels have been shown to increase in the livers of $CCl_4$-treated mice[11], as well as in chronically damaged human livers[173]. Moreover, the establishment of liver organoid cultures bearing the bipotential LSPCs requires activation of Wnt signaling by Wnt3a and R-spondin both in mice and humans[11,98].

**LIVER ORGANOIDS FOR STEM CELL BASED THERAPIES**

For end-stage liver diseases as well as for certain liver-based metabolic disorders, organ transplantation forms the only curative therapy[174]. However, currently liver organ transplantations cannot satisfy the demands due to the low number of available donors[174]. This prompted scientists to investigate cell therapy as an alternative treatment for restoration of deficient liver functions in patients. Several studies in animal models and people with chronic liver failure or liver enzyme-defects have shown that hepatocyte transplantation could possibly replace organ transplantation[175]. Besides this, owing to liver's well vascularized structure and capacity to secrete into the bloodstream, liver cell therapy has been indicated to be applicable for patients with enzyme deficiencies, including coagulation disorders and metabolic diseases[175].
For widespread applications of liver cell therapy, it is important to have high number of suitable liver cells. However, hepatocytes have proved to be difficult to culture, despite their remarkable proliferation capacity *in vivo*[176]. With respect to the utility of liver cells for therapeutic purposes including transplantations, the establishment of long-term liver organoid cultures from adult human liver has been a major step[98]. Owing to their high expandability, genetic stability and bipotentiality, liver stem cells in human organoids offer a safe source of liver cells for transplantation and subsequent treatment of a variety of diseases. Additionally, it is noteworthy that targeted genetic manipulation of liver stem cells is possible and important for gene therapy approaches. Furthermore, given the already

proven suitability of liver as an organ for gene therapies in treatment of metabolic disorders such as lysosomal storages disorders[177,178], it is tempting to investigate a liver organoid based gene therapy approach for treatment of such diseases.

**Lysosomal storage diseases (LSDs): Hurler disease**

With their content of more than 40 hydrolytic enzymes, lysosomes constitute the major site of digestion in a cell[179]. Therefore, mutations in genes that encode these hydrolytic enzymes or lysosomal membrane or accessory proteins lead to a blockage in degradation of macromolecular substrates[180]. The progressive accumulation of undigested metabolites results in cellular and tissue damage with subsequent multiorgan dysfunctions and, frequently, neurodegeneration. These systemic and clinically heterogeneous disorders, known as lysosomal storage diseases (LSDs), represent a family of more than 50 monogenic, heritable diseases and collectively affect 1 in 5000 new born children[180]. The age at onset of clinical symptoms and severity of LSDs vary, depending on the degree of residual functional protein, chemical properties of the stored macromolecule, and the cell types where storage takes place[181].
Mucopolysaccharidosis type I (MPS-I), which is caused by an inherited deficiency in the enzyme α-L-iduronidase (IDUA), constitutes one of the most frequent LSDs[182]. The most severe form of MPS I, known as Hurler syndrome, occurs mainly due to a homozygous or a compound heterozygous nonsense mutation in the IDUA gene[183]. Deficiencies in IDUA enzyme result in defects in the degradation of its substrates, glycosaminoglycans (GAGs). The accumulation of GAGs causes a variety of pathological processes in the skeletal, cardiac, digestive, respiratory, and central nervous systems[183]. Hurler disease manifestations include significant developmental delay, coarse facial features, skeletal dysplasia, cardiac insufficiency and hepatic disease along with cognitive decline and start as early as six months after birth[182]. Without treatment, the life expectancy of Hurler patients ranges between six to ten years.

**Treatment modalities for Hurler disease**

The current and potential therapies of Hurler disease, and other LSDs, which occur due to soluble enzyme deficiencies, are based on normal lysosomal enzyme trafficking and a phenomenon referred to as cross-correction. Newly synthesized hydrolytic enzyme precursors, including IDUA, are glycosylated in the ER and phosphorylated in the Golgi where they are recognized by their specific glycoprotein receptors and trafficked into lysosomes[184,185]. A small percentage of enzyme precursors escape from this route and are secreted to the extracellular matrix where they can be recovered via receptor-mediated endocytosis and targeted to lysosomes on neighboring cells, at near or distant sites[184,185]. This secretion-uptake or cross correction mechanism enables wild type enzymes to be taken up and targeted to lysosomes of otherwise enzyme-deficient cells.
Exploiting the cross-correction mechanism, current therapies of Hurler patients include enzyme replacement therapies (ERT) and hematopoietic stem cell (HSC) transplantations[183]. ERT involves repeated intravenous administrations of a recombinant IDUA enzyme into Hurler patients, and has been reported to have clinical benefits such as decreased hepatomegaly, improved pulmonary function and walking capacity[183]. However, since the half-life of the recombinant enzyme is short, ERT requires regular and life-long treatment of the patient

and is very costly[186]. Unlike ERT, HSC transplantations (using bone marrow, peripheral blood or UCB as a source) offer a widespread and long-lasting source of functional enzyme to the patients. Although HSC engraftment has been shown to lead to rapid reduction of GAG in liver, spinal fluid and urine[187], allogeneic HSC transplantation bears limitations and significant risks associated with the procedure itself such as finding a suitable donor and graft-vs-host disease, early morbidity and mortality.
Hurler disease, along with other LSDs, is a particularly good candidate for stem cell mediated gene therapy approaches for various reasons[184,186]. As Hurler disease is caused by a single gene defect, the correction of the gene can be relatively straightforward. Besides this, overexpression of lysosomal enzymes appears to be well tolerated[184,186] and even 5-10% of normal enzyme levels might be therapeutic. Moreover, several studies in mice models have suggested that HSC transplantation could be more successful when coupled to gene therapy by which higher levels of the enzyme could be achieved[188–190]. Nevertheless, the use of HSCs for *ex vivo* gene therapy approaches retains the same hurdles related to HSC transplantations. Moreover, it is not possible to apply targeted modifications in HSCs and to eliminate the risk of insertional mutagenesis[184].
Given the fact that the liver serves as a good depot organ for secretion of enzymes, the recently established adult liver organoids can be tested as a stem cell source for genetic modifications, as an alternative to *ex vivo* gene therapy directed to the HSCs. Exploitation of this approach might offer an additional therapy modality which could provide Hurler patients with a safe and long-lasting source of IDUA enzyme.

**AIMS OF THE THESIS**

Due to their biological properties and medical value, stem cells are a subject of increasing interest for basic research. With regards to the applicability of stem cells for therapy related purposes, the identification of the molecular pathways involved in the regulation of stem cell activity *in vitro* is especially necessary. Over the years, Wnt signaling molecules have been implicated in the control of stem cells from a variety of tissues. Therefore, the studies in chapters 2-5 of this thesis aim to assess the role and use of Wnt signaling proteins for expansion of stem cells *in vitro* from multiple mammalian tissues including intestine, liver and blood, for enhancing the applicability of adult stem cells in clinical purposes.

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# CHAPTER 2

# Lipid-mediated stabilization of WNT ligands removes dual impediment to serum-free culture of human organ stem cells

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# CHAPTER 3

# Wnt3a reduces the number of mouse hematopoietic stem and progenitor cells in stroma-free, serum-free cultures

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**Abstract**

The Wnt signaling pathway has been implicated to play a role in the regulation of mouse hematopoietic stem and progenitor cell (HSPC) fate decisions between self-renewal and differentiation. However the role of Wnt proteins in *ex vivo* culture of HSPC remains controversial. Here, we show that while Wnt signals have no apparent effect on the number and functionality of mouse $Lin^-$, $Sca1^-$, and $c\text{-}Kit^+$ (LSK) cells in stroma-supported cultures, it leads to a reduction in the number of these cells in a stroma-free, serum-free culture system. The same negative effect on the number of LSK, $CD48^-$, $CD150^+$, $CD34^{low}$ cells was observed even when a more stable Wnt signal, provided in the form of a lipid-stabilized Wnt3a protein or by the GSK3-β inhibitor CHIR99021, was used. The decrease in mouse LSK number occurred at later stages of the culture. Overexpression of the anti-apoptotic BCL2 protein did not prevent the Wnt-induced loss of LSK, $CD48^-$, $CD150^+$, $CD34^{low}$ cells, suggesting that the loss was not due to apoptosis. These data suggest that Wnt signals impede the maintenance of HSPC in stroma- and serum-free cultures.

## Introduction

Wnt signals have been shown to act as self-renewal factors for both embryonic[1] and adult stem cells in a diversity of mammalian tissues, including intestines, skin and mammary gland[2]. They also provide critical cues for maintenance or expansion of stem cells in cultures of a variety of adult stem cells both from mouse and human[3–8]. Several signal transduction cascades may be induced upon binding of Wnt ligands to their receptors[9], the best-characterized one being the canonical or β-catenin pathway. In this pathway, β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK-3β) to be targeted for proteasomal degradation. Upon binding of a Wnt protein to the Frizzled (Fz) family of receptors and to low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6 co-receptors, the phosphorylation of β-catenin is inhibited. Stabilized β-catenin translocates into the nucleus where it interacts with TCF/LEF transcription factors to activate targeted gene expression[10]. Over the past decades, a variety of studies have implicated a possible role for canonical Wnt signaling in the control of mammalian hematopoietic stem and progenitor cell (HSPC) function. Mice carrying a null allele of Wnt3a[11] or overexpressing the Wnt inhibitor *Dickkopf1* in the bone marrow niche[12] display impaired hematopoietic stem cell (HSC) self-renewal. When stimulated by a constitutively active form of β-catenin or by purified Wnt3a protein, Wnt signaling increased self-renewal capacity of apoptosis-resistant, transgenic HSPC *ex vivo*[13,14]. However, mouse models with constitutively activated Wnt signalling displayed a multilineage differentiation block and loss of HSCs[15,16], and later studies using purified Wnt proteins in *ex vivo* cultures of normal mouse HSPC reported controversial outcomes. In one study, Wnt5a was suggested to improve repopulation capacity of mouse HSPC by inhibiting canonical Wnt pathway induced by Wnt3a protein[17], while in another it was shown to impede maintenance of HSPC[18]. Recently, we showed that purified Wnt3a protein-mediated activation of canonical Wnt signaling reduces expansion of human umbilical cord blood (UCB)-derived HSPC by inducing their differentiation[19]. Several other studies showed that active β-catenin induces apoptosis in HSPC[20,21]. Taken together, these studies underscore the controversies on the effect of Wnt activation on HSPC proliferation, differentiation and apoptosis.

It has been known for some time that Wnt proteins have a very short half life in serum-free cell culture media[22], losing its activity within several hours[19,22]. This complicates the interpretation of the results obtained for the role of Wnt signals on ex *vivo* HSPC proliferation. Thus, addition of Wnt3a protein to cell cultures would result in intermittent rather than continuous activation of the pathway, which may be insufficient to inhibit HSPC differentiation. In this study, we revisited the effect of Wnt signals on *ex vivo* cultures of mouse HSPC and investigated in more depth the role of apoptosis and of Wnt3a protein stability on the response of the cells to Wnt signals.

## Methods and Materials

### Mice

C57BL/6 and TRE-BCL2 mice used in this study were bred and maintained in the animal facility at Erasmus Medical Center (Erasmus MC), and handled according to institutional guidelines. All procedures were carried out in compliance with the Standards for Care and

Use of Laboratory Animals. All mice were used at 12-24 weeks of age.
For generation of TRE-BCL2 mice, a human BCL2 cDNA (accession number BC027258) was amplified, and a Kozak sequence and C-terminal FLAG tag added. The vector p2Lox.GFP[23] was digested with XhoI and NotI to remove the GFP sequence, and the XhoI/NotI-digested BCL2-FLAG sequence ligated into its place to create p2Lox.BCL2. 25 µg of p2Lox.BCL2 was electroporated into A2Lox.Cre ES cells, followed by induction of the cells with 1 µg/ml doxycycline overnight to induce recombination, and the cells selected on 250 µg/mL of G418, as previously described[24]. The cells were then used for blastocyst injections into C57Bl/6 blastocysts. Chimeras were mated against C57Bl/6 mice and readily gave germline transmission. Mice containing the BCL2 transgene and the reverse tetracycline transactivator (rtTA, expressed from the Rosa26 locus in A2Lox.Cre ES cells) were identified by PCR using the following primers:
BCL2 forward: GGATGCCTTTGTGGAACTGT;
BCL2 reverse: GCTCACTTGTCGTCATCGTC;
rtTA forward: GGACGAGCTCCACTTAGACG ;
rtTA reverse: GGCATCGGTAAACATCTGCT.

**Isolation of LSK and LSK CD48- cells**

For LSK or LSK CD48$^-$ cells isolation, femurs and tibias from mice were removed, BM flushed, and mononuclear cells were isolated by Lymphoprep™ according to manufacturer's instructions (Stem Cell Technologies, Grenoble, France). Lineage-positive (Lin$^+$) cells were depleted by magnetic cell sorting (MACS) with the use of a lineage cell depletion kit according to manufacturer's instructions (Miltenyi Biotech, GmBH, Bergisch Gladbach, Germany). Lin depleted cells were further stained with a Lin-PE cocktail (anti-CD3e-PE, anti-CD45R-PE, anti-CD11b-PE, anti-Gr1-PE, and anti-Ter119-PE) as well as with anti-c-Kit-APC and anti-Sca-1-PE-Cy7 (all from BD Pharmingen, Heidelberg, Germany) where indicated. Hoechst (BD Biosciences, San Jose, CA, USA) was added to allow live/dead cell discrimination. Cell sorting for Lin$^-$, c-Kit$^+$, Sca-1$^+$ (LSK) cells was performed using BD FACSAriaIII™ (BD Biosciences, San Jose, CA, USA).
For sorting LSK CD48$^-$ population, cells were stained with Lin-PE cocktail, anti-CD48-PE (BD Pharmingen, Heidelberg, Germany) anti-c-Kit-APC-Cy7 (eBioscience, Vienna, Austria) and anti-Sca-1-PE-Cy7. For further analysis of the starting population, these cells were also stained with CD34-FITC (BD Pharmingen, Heidelberg, Germany), and/or CD150-APC (Biolegend, London, UK) where indicated.

**Co-culture of LSK with stromal layer**

UG26-1B6 cells were maintained as previously described[25]. For co-cultures with LSK cells, stromal cells were grown on 0.1% gelatin (Sigma, St. Louis, MO, USA) coated 6-well plates (Corning, Lowell, MA, USA). Once cells reached confluence, they were irradiated at 20 Gy. 1500 FACS-sorted LSK cells were cultured on stroma at 33°C and 5% $CO_2$. The cells were cultured in 2 ml of long-term culture (LTC) medium (MyeloCult M5300, Stem Cell Technologies, Grenoble, France), which contains horse and fetal bovine serum with hydrocortisone ($10^-6$ M, Stem Cell Technologies, Grenoble, France). 250 ng/ml purified Wnt3a or 2 µM IWP2 or both were added to LTC medium, and half the medium was refreshed every three days for 2 weeks. After 2 weeks, cells were harvested for FACS analysis and CFU assays.

**Colony-forming unit (CFU) assay**

Fresh or culture progeny were plated at various dilutions in triplicate in methylcellulose semi-solid medium (MethoCult GF m3434, Stem Cell Technologies Inc., Grenoble, France) with with 100 U/ml Penicillin and 100 µg/ml Streptomycin (both Invitrogen, Life Technologies, Bleiswijk, The Netherlands). All cultures were incubated at 37°C and 5% $CO_2$ for 12 days. Hematopoietic colony types (erythroid-burst-forming unit (BFU-E), colony-forming unit-granulocyte (CFU-G), colony-forming unit-macrophage (CFU-M), colony-forming unit-granulocyte, macrophage (CFU-GM) and colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM)) were distinguished by morphology and counted with an inverted microscope.

**Stroma-free, serum-free cultures**

Sorted LSK or LSK CD48$^-$ cells were cultured in StemSpan™ Serum-Free Expansion Medium (SFEM, Stemcell Technologies, Grenoble, France) supplemented with 1% penicillin/streptomycin (Sigma, St Louis, MO, USA), 10 µg/ml low molecular weight heparin (Abbott, Wiesbaden, Germany), SCF (10 ng/ml, Biovision, Milpitas, CA, USA) and TPO (20 ng/ml, Cell Sciences, Canton, MA, USA). Cells were cultured in a volume of 200 µl medium in a U-bottom 96-well plate at a range of 3000-30.000 cells/ml at 37 °C in 5% CO2. Where indicated purified Wnt3a (250 ng/ml, if not indicated otherwise), liposomal Wnt3a (250 ng/ml), Wnt antagonist Fz8CRD (5 µg/ml) and CHIR99021 (1uM, Stemgent Cambridge, MA, USA) were added to the cultures daily by half medium refreshment or splitting.
To induce BCL2 expression in sorted cells derived from TRE-BCL2 mice, doxycycline (1µg/ml, Sigma, St Louis, MO, USA) was added to cultures at the start of the experiment as well as during the refreshments or splitting.

**FACS analysis of cultures**

For analysis of cultures after the indicated culture period, cells were harvested, washed and resuspended in PBS containing 10% FCS. After co-cultures, total cells were counted in trypan blue on a Neubauer hemocytometer. For stroma-free, serum free cultures, absolute numbers of viable HSPC populations (LSK, LSK CD48$^-$, and LSK, CD48$^-$, CD150$^+$, CD34$^{low}$) cells were determined by a single platform flow cytometric analysis, using the same panel of monoclonal antibodies that was used for sorting and a calibrated number of Cyto-Cal™ Control Count counting microspheres (Thermo Scientific, Waltham, MA, USA).
For apoptosis analysis, cells were simultaneously stained with anti-Annexin V-APC in binding buffer (BD Biosciences, San Jose, CA, USA) in PBS containing 10% FCS. Flow cytometric analysis was performed using a BD FACSFortessa™ (BD Biosciences, San Jose, CA, USA) and data was analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

**Purification of Wnt3a protein and preparation of liposomal Wnt3a**

Mouse Wnt3a was purified from Wnt3a-conditioned medium, collected from *Drosophila* S2 cells, using Blue Sepharose affinity and gel filtration chromatography as described previously[14].
Liposomes containing DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), DMPG

(1,2-dimyristoyl-sn-glycero-3-phospho-rac-glycerol) (both Lipoid AG, Ludwigshafen, Germany) and Cholesterol (Sigma, St Louis, MO, USA) at a 10:1:10 molar ratio were prepared as described previously[19]. Briefly, purified Wnt3a was mixed with liposomes and incubated for one hour by rotating at 4°C. CHAPS was removed by dialysis with a membrane with molecular weight cut-off of 10 kDa (Thermo Scientific, Waltham, MA, USA) in HBS at 4°C. The Wnt liposomes were stored at 4°C. Activity of purified Wnt3a protein and liposomal Wnt3a were determined in a luciferase reporter assay as described previously[19].

**Luciferase reporter assay**

Mouse LSL cells, which express luciferase in response to TCF promoter binding[26], were routinely cultured at 37°C and 5% $CO^2$ in culture medium containing DMEM (Invitrogen, Life Technologies, Bleiswijk, The Netherlands), 10% FCS (HyClone, Thermo Scientific, Waltham, MA, USA), 100 U/ml Penicillin and 100 µg/ml Streptomycin (both from Invitrogen, Life Technologies, Bleiswijk, The Netherlands). Prior to the activity assays, 25,000 LSL cells were plated in each well of a 96-well plate and grown for 24 hours. Wnt3a reagents were separately incubated in culture medium with or without serum for various periods of time at 37°C in 96-well plates. After the incubation intervals, media with Wnt3a reagents were added to LSL cells. Upon an additional overnight incubation with the indicated reagents, relative luciferase units were measured with a Glomax multiplate reader.

**Western blotting**

Lineage-negative ($Lin^-$) cells were isolated by FACS sorting as described above. 100,000 cells were seeded for each condition and were cultured for 24h with and without doxycycline (1µg/ml, Sigma, St Louis, MO, USA) treatment. Whole-cell extracts were then isolated from total cells. Cells were washed twice in PBS and lysed in RIPA buffer (Thermo Scientific, Waltham, MA, USA) including protease and phosphatase inhibitors. Protein concentrations were measured by BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) and equal amounts of protein were subjected to SDS-PAGE, followed by western blot analysis using anti-Flag antibody (1:1500, Sigma, St Louis, MO, USA).

**Statistical analysis**

Data are presented as mean ± SD. Statistical significance was detemined by a Student's two-tail t-test (unpaired and ratio-paired), performed using GraphPad Prism software (version 6.0; GraphPad), and $p<0.05$ was defined as statistically significant.

**Results**

**Effect of exogenous and endogenous Wnt signals in stroma-supported LSK cultures**

Previously, a murine urogenital-ridge derived stromal cell line, UG26-1B6, was shown to induce proliferation and differentiation of mouse progenitor cells and support maintenance of mouse HSC [25,27]. Wnt5a, which is expressed by this stromal layer, has been suggested to be one of the factors involved in this maintenance[27]. To assess whether exogenous Wnt3a protein enhances UG26-1B6 stroma-mediated effects on HSPC, we co-cultured adult mouse bone

marrow Lin$^-$, c-Kit$^+$, Sca-1$^+$ (LSK) cells, which are enriched for HSPC[28], on confluent, irradiated UG26-1B6 stromal cell layers in the presence of purified Wnt3a protein. We observed no change in total cell numbers in response to the Wnt3a treatment (Figure 1A, p=0.56). FACS analysis of 2-week progeny demonstrated that the majority of cells were differentiated, regardless of the presence of exogenous Wnt3a (Figure 1B). Although Lin- cells did expand in our co-culture system regardless of the absence or presence of exogenous purified Wnt3a (Figure 1C, p=0.51), only a small percentage of cells retained the c-Kit and Sca-1 markers, constituting the HSC enriched LSK population (Figure 1D). As a consequence, the number of LSK cells cultured with purified Wnt3a also remained similar to control condition (Figure 1E, p=0.40).



To test whether we achieved stable Wnt activity in our co-culture system, we incubated purified Wnt3a protein for various periods of time in the co-culture medium (LTC medium containing serum) at 37°C and assayed the remaining activity using the LSL reporter assay[26]. LSL cells contain a Wnt responsive luciferase reporter, allowing a quantitative readout of Wnt activity. This showed that purified Wnt3a protein retained its activity for over three days in LTC medium (Figure 1F). This suggests that the lack of effect of Wnt3a protein was not due to absence of activity.

To address whether endogenous Wnt proteins, produced by the stromal cells, played a role in the UG26-1B6 stroma-mediated effects on LSK cells, we inhibited their production using the small molecule inhibitor IWP2[29]. Addition of IWP2 to co-cultures of irradiated UG26-1B6 cells and LSK cells had little effect on expansion of total cells (Figure 1A, p=0.23), or on the frequency (Figure 1B) and expansion of Lin- cells (Figure 1C, p=0.96). Furthermore, inhibition of endogenous Wnt proteins did not significantly change the frequency of c-Kit$^+$, Sca-1$^+$ cells (Figure 1D) or the number of LSK cells (Figure 1E, p=0.92).

To further evaluate the impact of Wnt signals on the proliferation and differentiation of progenitors in our co-cultures, we performed CFU assays. The total number of CFUs slightly increased compared to input in all conditions (Figure 1G). However, we found no significant differences in the frequencies of BFU-E, CFU-G, CFU-M, CFU-GM, or CFU-GEMM regardless of addition of Wnt3a protein or inhibition of Wnt production (Figure 1G). Collectively, these results suggest that neither exogenous Wnt3a activity nor inhibition of endogenous Wnt proteins plays a role in maintenance of LSK cells in co-culture with UG26-1B6 stromal cells.

**Wnt/beta-catenin signals reduce the number of mouse LSK cells in stroma- and serum-free cultures**

Since the complex nature of stroma – LSK co-culture systems might hamper the evaluation of the role of Wnt3a, we assessed its effect in stroma- and serum-free culture of mouse HSPC. For this, FACS-sorted LSK cells were cultured for 10 days in serum-free medium containing stem cell factor (SCF) and thrombopoietin (TPO). Addition of Wnt3a protein did not significantly affect the expansion of total nucleated cells in this culture system (Figure 2A). However, treatment with purified Wnt3a protein resulted in a 2-fold decrease in the number of Lin$^-$ cells relative to control condition (Figure 2B, p<0.01). Moreover, while approximately half of the LSK cells were maintained in control conditions, treatment with Wnt3a led to a 6-fold decrease in the number of LSK cells (Figure 2C and 2D, p=0.01). These effects were reversed upon simultaneous addition of Fz8CRD, a soluble domain of the Wnt receptor that sequesters Wnt proteins[30], indicating they were specific for the Wnt3a protein (Figure 2A-D). A similar effect on the number of total nucleated cells, Lin- cells and LSK cells was observed

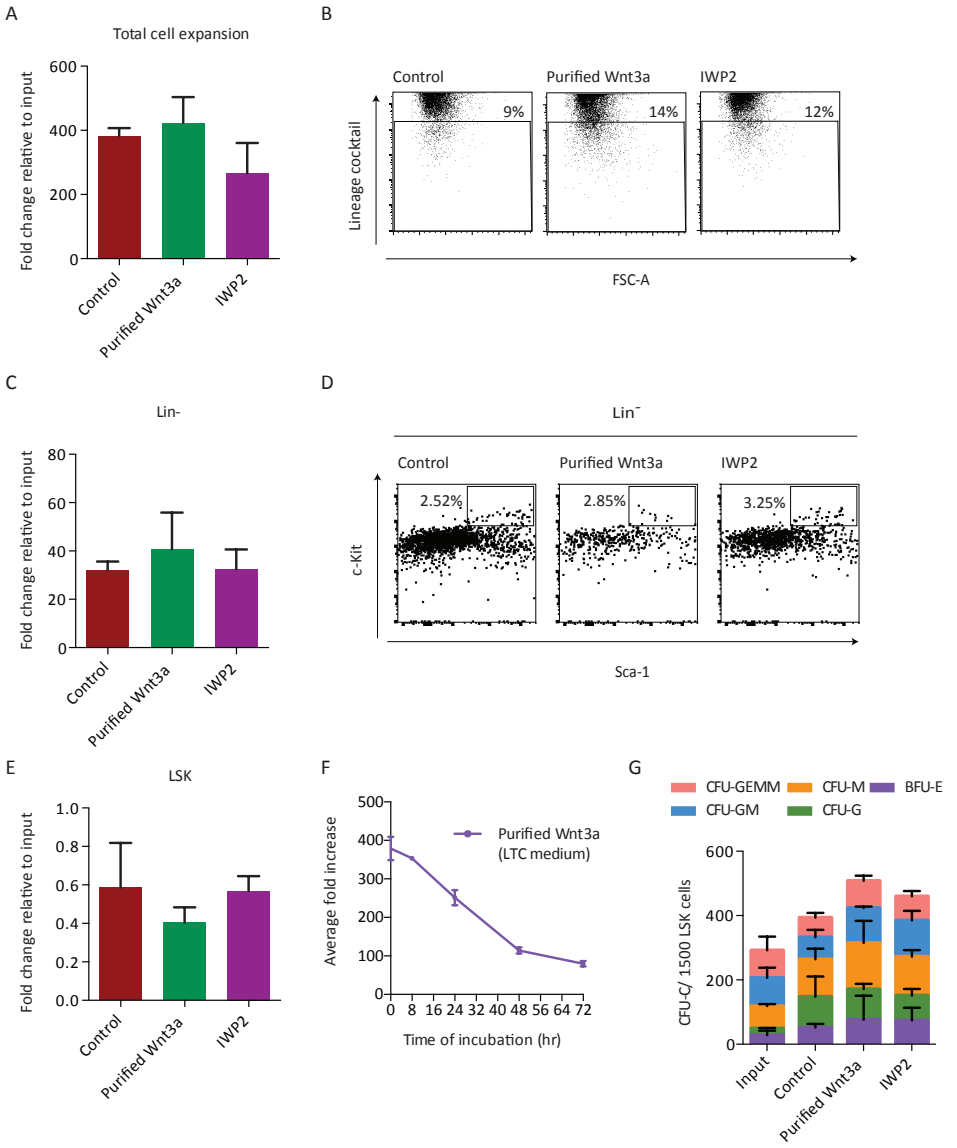


**Figure 1. Wnt signals do not promote expansion of LSK cells in UG26-1B6 stromal cell co-culture**
1500 LSK cells were co-cultured with UG26-1B6 that had been irradiated at 20 Gy. After 2 weeks of culture, (A) total cell number was enumerated to determine cell expansion. (B) Representative plots of FACS analysis of the progeny for lineage markers. Percentages of Lin$^-$ cells among viable cells (gated region) are shown. (C) Quantification of Lin$^-$ cells fold expansion after culture in the presence of indicated molecules. (D) Lin$^-$ cells were further analyzed for c-kit and Sca-1 markers. Percentages of c-kit$^+$, Sca-1$^+$ cells among Lin$^-$ population (gated region) are shown. (E) Fold change of LSK relative to input is calculated. (F) Quantification of Wnt activity retained after incubation for 0, 8, 24, 48 and 72 hours at 37°C in co-culture medium (LTC medium). Activity plot displays average increase of luminescence relative to background. Purified Wnt3a was added at 250 ng/ml (Error bars indicate S.E.M., n=3). (G) Progeny of co-cultures were plated in CFU assay. Plotted are absolute numbers of BFU-E, CFU-G, CFU-M, CFU-GM and CFU-GEMM. (Error bars indicate S.D.) (n=2).

when the canonical Wnt pathway was activated using the GSK3 inhibitor CHIR99021 (Figure 2A-D), indicating that the effect was specific for the β-catenin pathway. The frequency of $Lin^+$ cells was not significantly affected by Wnt3a protein (Figure 2E, p=0.09). However, we observed a higher frequency of Lin+ cells when cells were induced with CHIR99021, which might be due to a stronger or more prolonged activation of the Wnt pathway, or by effects other than Wnt/β-catenin induction (Figure 2E, p<0.05). These data collectively show that the addition of Wnt3a protein decreased the number of LSK cells in serum-free, stroma-free cultures and that this is a consequence of the binding of Wnt3a to its receptor and subsequent activation of the canonical Wnt pathway.

Recent data indicates that stability of purified Wnt proteins depends on the presence of detergent or serum[22], implying that its activity may be rapidly lost in serum-free culture systems. This may explain its inability to support HSPC in serum-free culture. Luciferase reporter assays demonstrated that purified Wnt3a protein, while retaining its activity for several days in medium containing serum, loses its activity within a few hours in serum-free medium (Figure 3A). Even daily addition of purified Wnt3a to serum-free cultures would therefore result in brief pulses of Wnt3a activity instead of a sustained Wnt signal, which may be insufficient to support HSPC.



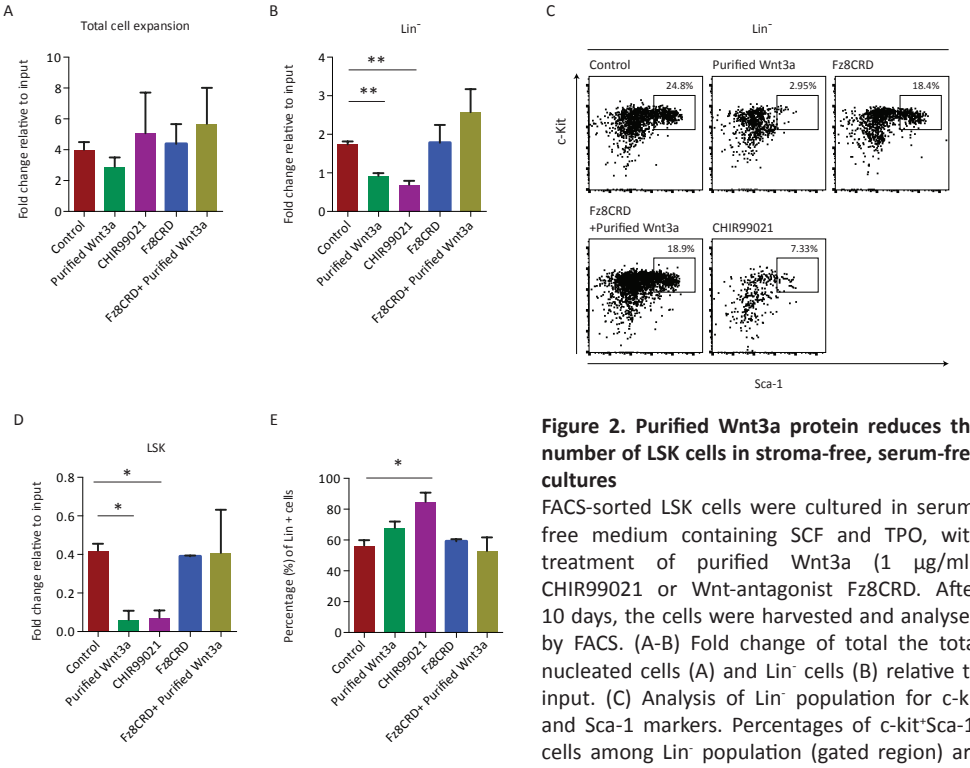


**Figure 2. Purified Wnt3a protein reduces the number of LSK cells in stroma-free, serum-free cultures**

FACS-sorted LSK cells were cultured in serum-free medium containing SCF and TPO, with treatment of purified Wnt3a (1 µg/ml), CHIR99021 or Wnt-antagonist Fz8CRD. After 10 days, the cells were harvested and analysed by FACS. (A-B) Fold change of total the total nucleated cells (A) and $Lin^-$ cells (B) relative to input. (C) Analysis of $Lin^-$ population for c-kit and Sca-1 markers. Percentages of $c\text{-}kit^+Sca\text{-}1^+$ cells among $Lin^-$ population (gated region) are shown. (D) Fold change of LSK relative to input is plotted. (E) The frequency of $Lin^+$ cells within total cells. (Error bars indicate S.D.) (n=2). Statistical significance was determined by a two-tail t-test. (*p<0.05).

We recently showed that association with lipid vesicles prolongs the stability of Wnt ligands[19,31,32], allowing us to achieve a more sustained Wnt signal in serum-free culture (Figure 3B). We therefore investigated whether lipid-stabilised Wnt3a protein would be able to support LSK, CD48$^-$ cells in stroma-free, serum-free conditions. After 7 days of culture, we observed no significant differences between regular and liposomal Wnt3a for total nucleated cell expansion (Figure 3C), and the frequency of Lin$^+$ CD48$^+$ cells remained similar in all conditions (Figure 3D). Moreover, like purified Wnt3a, liposomal Wnt3a induced a decrease in the more immature LSK, CD48$^-$, CD150$^+$, CD34$^{low}$ population, which is highly enriched for HSCs (Figure 3E, $p<0.05$ and $p<0.01$ for purified and liposomal Wnt3a, respectively). Thus, the failure of purified Wnt3a to support mouse HSPC in our serum-free culture system was not due to insufficient stability of the Wnt ligand since it also occurred in the presence of lipid-stabilized Wnt3a protein.

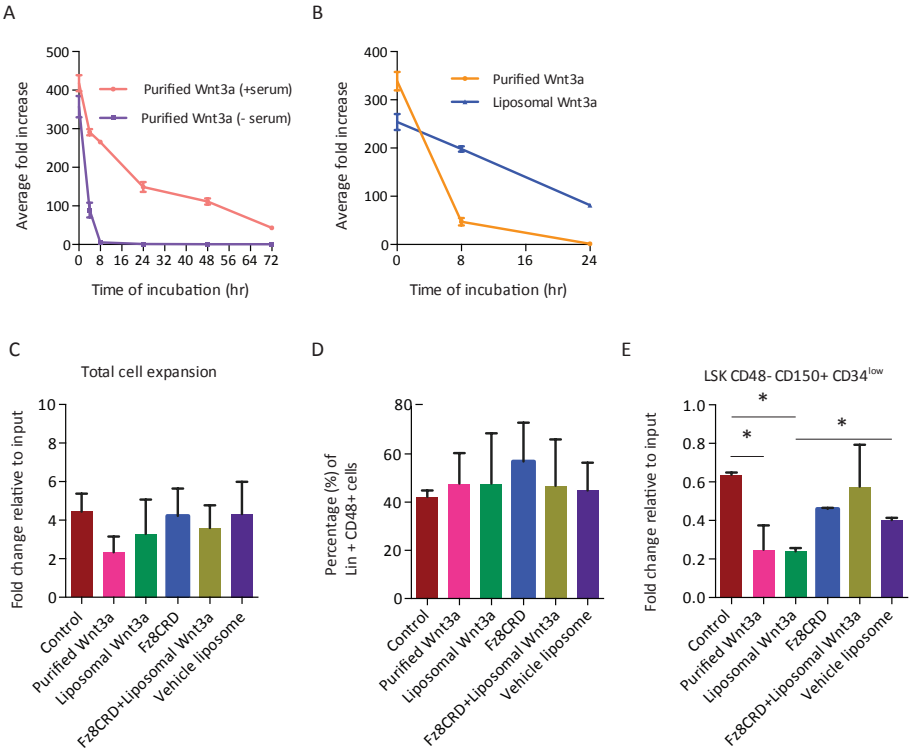


**Figure 3. Stable Wnt activity by liposomal Wnt3a reduces the number of primitive LSK cells *in vitro***
(A) Quantification of Wnt activity retained after incubation for 0, 8, 24, 48 and 72 hours at 37°C in cell culture media with or without serum. Activity plot displays average increase of luminescence relative to background. Purified Wnt3a was added at 250 ng/ml (Error bars indicate S.E.M., n=6). (B) Quantification of Wnt3a activity retained after incubation of the indicated reagents in serum-free cell culture medium at 37°C for 0,8 and 24 hours. Purified and liposomal Wnt3a were added at 250 ng/ml. (Error bars indicate S.E.M., n=10). (C-E) FACS sorted LSK CD48$^-$ cells, were cultured with purified Wnt3a, liposomal Wnt3a, and Fz8CRD. Total cells were analysed by FACS after 7 days of culture. The graphs display the total nucleated cell expansion relative to input (C), the frequency of Lin$^+$ cells within total cells (D), and the fold change of LSK CD48$^-$CD150$^+$CD34$^{low}$ cells compared to input (E). (Error bars indicate S.D.) (n=2). Statistical significance was determined by a two-tail t-test (*:$p<0.05$; **:$p<0.01$).

### Wnt3a signaling does not induce apoptosis in short-term cultures

Previous studies suggest that Wnt signals may induce apoptosis in HSPC, and that combined activation of Wnt signaling and inhibition of apoptosis may be required to support HSC expansion[17,20]. To assess whether Wnt activation leads to early apoptosis, we cultured FACS-sorted LSK CD48$^-$ cells in our stroma-free, serum-free system. After 4 days of culture, we did not observe any difference in total cell numbers or in the frequency of Hoechst$^-$, Annexin V$^+$ early apoptotic cells (Figure 4A-C). In contrast to longer-term cultures, as described above, there was no difference in the numbers of LSK CD48$^-$ and LSK CD48$^-$ CD34$^{low}$ cells when cultured with purified Wnt3a in comparison to control (Figure 4D-E). Moreover, we observed no increase in levels of early apoptotic LSK cells in the presence of Wnt3a (Figure 4F-G). This suggests that Wnt-mediated reduction in the number of LSK occurs at later stages of culture and cannot be explained by an increase in early apoptotic events.

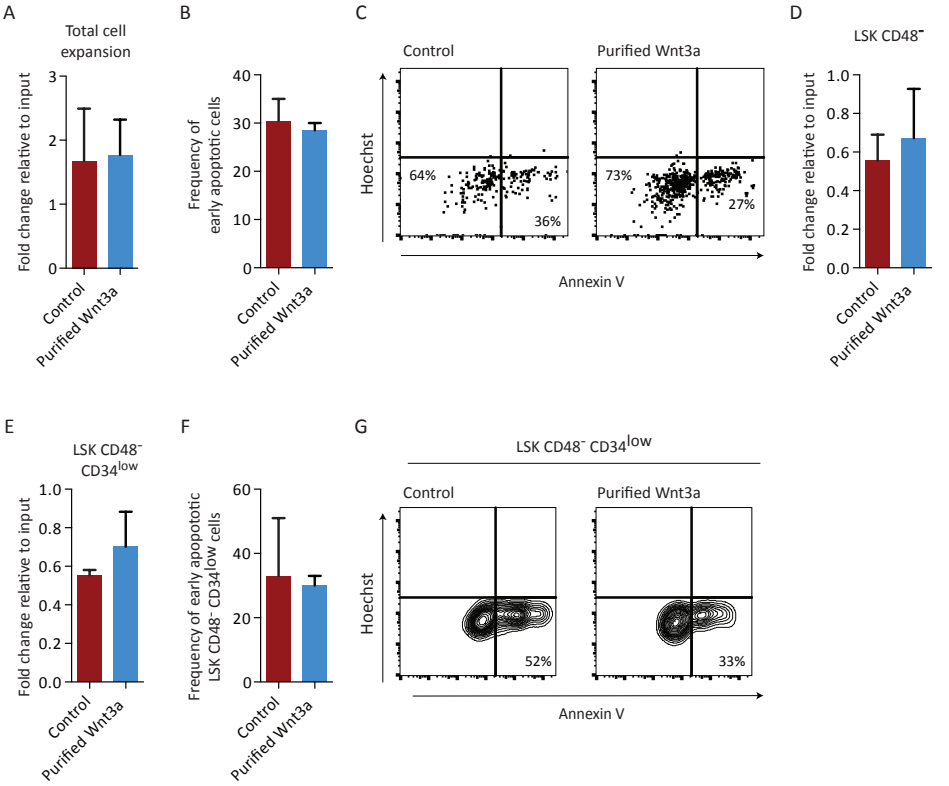


**Figure 4. Wnt3a protein does not induce apoptosis of LSK cells in short-term stroma-free, serum-free cultures**

FACS-sorted LSK cells were cultured in our serum-free conditions with the addition of purified Wnt3a. Cells were analysed by flow cytometry after 4 days of culture. (A-B) The graphs display fold change of total nucleated cells (A) and the frequency of Annexin-V$^+$Hoechst$^-$ early apoptotic cells among the same population (B). (C) Representative FACS plots for Annexin-V analysis within Hoechst$^-$ viable total cells. Percentages of Annexin-V$^-$Hoechst$^-$ and Annexin-V$^+$Hoechst$^-$ cells are shown. Shown are graphs for fold change of LSK CD48$^-$ (D), and LSK CD48$^-$CD34$^{low}$ cells (E). (F) The graph displays the frequency of Annexin-V$^+$Hoechst$^-$ early apoptotic LSK CD48$^-$CD34$^{low}$ cells among the same population. (G) Representative FACS plots for Annexin-V analysis within LSK CD48$^-$CD34$^{low}$ cells. Percentages of Annexin-V$^+$Hoechst$^-$ cells are shown. (Error bars indicate S.D.) (n=2).

**Inhibition of apoptosis does not rescue Wnt3a-induced loss of LSK cells**

To further investigate a possible role for apoptosis in Wnt-induced loss of HSPC in our cultures, we developed a novel transgenic anti-apoptotic mouse model. Using Cre-mediated transgenesis in embryonic stem cells, we placed an anti-apoptotic FLAG-tagged human BCL2 transgene under control of a doxycycline-responsive promoter targeted in the HPRT locus. The reverse tetracycline transactivator was constitutively expressed from the Rosa locus. Thus, the cells could be induced to express BCL2 upon treatment with doxycycline, inhibiting the induction of apoptosis. Transgenic mice were generated using blastocyst injection of the cells, which allowed us to obtain LSK cells carrying the transgenes. We verified that doxycycline indeed induced BCL2 in primitive hematopoietic $Lin^-$ progenitors by Western blot analysis (Figure 5A). This system enabled us to investigate the effect of BCL2 overexpression in otherwise fully comparable cell populations.
Doxycycline treatment substantially enhanced total nucleated cell expansion upon culture of LSK $CD48^-$ cells for 10 days (Figure 5B, $p<0.05$), confirming the effectivity of BCL2 induction. Moreover, a strong increase in LSK $CD48^-$ cells and of the more immature LSK $CD48^-$ $CD150^+$ $CD34^{low}$ population was observed upon doxyxycline induction (Figure 5C and 5D, $p<0.05$ and $p<0.01$, respectively), indicating that apoptosis was indeed an important factor limiting the expansion of these cells in culture. However, with combined doxycycline and liposomal Wnt3a treatment, the total nucleated cell expansion was reduced compared to doxycycline only condition (Figure 5B, $p<0.05$). Likewise, the expansion of LSK $CD48^-$ cells and of LSK $CD48^-$ $CD150^+$ $CD34^{low}$ cells in response to BCL2 induction was repressed by liposomal Wnt3a treatment (Figure 5C, $p<0.05$, and Figure 5D, $p<0.01$ and $p<0.001$). Combined, our data showed no evidence that Wnt3a induced early apoptosis in HSPC, or that inhibition of apoptosis by BCL2 supported HSPC expansion in response to Wnt3a protein.

**Discussion**

In this study, we investigated the role of Wnt3a-induced signaling in regulation of mouse HSPC *ex vivo*. While purified Wnt3a protein showed little impact on the number and functionality of LSK cells when cultured in a stromal context, it induced a decrease in the number of HSPC in a stroma-free, serum-free culture system. This effect was reversed by a competitive Wnt antagonist, indicating that it was due to binding of Wnt3a to its receptor. We explored the hypothesis that the failure of Wnt3a protein to support HSPC in a serum-free context was due to its very short half-life in these conditions, and developed a lipid-stabilized Wnt3a formulation able to provide a sustained Wnt signal. However, lipid-stabilized Wnt3a protein induced a similar reduction in the number of HSPC. In addition, GSK3-b inhibition also resulted in a similar reduction of HSPC number, strongly suggesting that the effect was due to activation of the canonical Wnt pathway. Finally, we investigated whether the induction of apoptosis by Wnt signals caused the loss of HSPC. However, we found no evidence that Wnt signals induced apoptosis. Moreover, we developed a transgenic mouse model in which apoptosis can be inhibited by the inducible expression of BCL2. While on its own this greatly supported the expansion of HSPC, it did not prevent the loss of HSPC in response to Wnt signals. Combined, these data indicate that Wnt signals do not support expansion of mouse HSPC in stroma-free serum-free culture but instead induce loss of these cells. Since the loss was not mediated by apoptosis, the most likely explanation would be that Wnt signals induce differentiation of the cells.

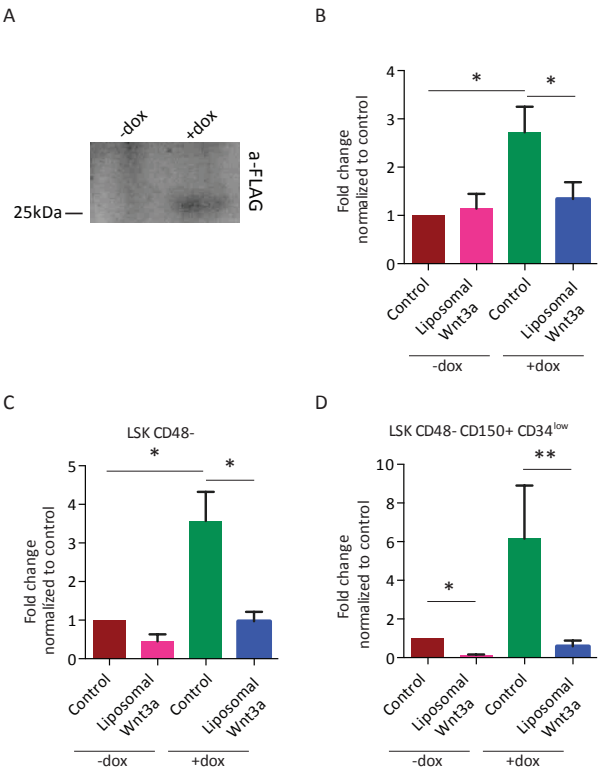


**Figure 5. The negative effect of Wnt signals on LSK cell number is not reversed by BCL2 overexpression**
(A) Western blotting for FLAG protein in Lin$^-$ cells cultured for one day with doxycyline treatment. (B-D) LSK CD48$^-$ cells from TRE-BCL2 mice were cultured with and without liposomal Wnt3a and doxycyline treatment for 10 days. The graphs display fold change of the total nucleated cells (B), LSK CD48$^-$ (C) and LSK CD48$^-$ CD150$^+$CD34$^{low}$ cells (D) normalized to control. (Error bars indicate S.D.) (n=3). Statistical significance was determined by a two-tail t-test. (*p<0.05, ** p<0.01 and 0.001).



A number of studies indicate an important role for Wnt ligands in the maintenance/expansion of HSC *in vitro*[13,14,27]. For instance, addition of anti-Wnt5a antibodies to inhibited the repopulation capacity of HSCs, suggesting that Wnt5a protein is necessary for maintenance of HSPC in these co-cultures[27]. However, we observed that inhibition of endogenous Wnt protein production by IWP2 in UG26-1B6 co-cultures affected neither the number of LSK cells nor the number of CFU. This discrepancy might be due to the use of antibodies by Buckley *et al*., which might induce other effects, such as provoking an immune response, acting in the bone marrow niche, or interfering with homing capacity. Moreover, addition of exogenous Wnt5a to the UG26-1B6 co-cultures did not impact the repopulation capacity of HSCs[27]. Similarly, we did not observe an effect of Wnt3a proteins on the number of HSPC or CFU, suggesting that Wnt ligands do not impact on the expansion of HSPC in this co-culture system. Nevertheless, a co-culture system containing serum and growth factor-producing stromal cells, which is highly differentiation inducing (see Figure 1B), might mask differentiation-inducing effects of Wnt signals on HSPCs.

Willert *et al*. and Reya *et al.* report that Wnt signaling activation by either purified Wnt3a protein or a constitutively active form of β-catenin *in vitro* induced expansion of mouse BCL2-overexpressing HSPC[13,14]. In contrast, Nemeth *et al*. observed that activation of Wnt signaling by purified Wnt3a proteins induced a decrease in the number of wild-type mouse LSKI (Lineage negative, Sca-1$^+$, c-kit$^+$, IL-7Rα$^-$) cells during culture in serum-free medium[17]. However, this study did not account for the rapid loss of Wnt protein activity that occurs in the absence of serum[22]. We show here that lipid-stabilized Wnt3a protein, which retains activity for more than 24 hrs in the absence of serum, also induced loss of mouse HSPC.

Moreover, we previously showed that the number of human HSPC was also reduced upon induction of Wnt signaling via purified or lipid-vesicle stabilized Wnt3a proteins[19]. Several *in vivo* studies indicated increased apoptosis of HSPC by activated Wnt signaling, suggesting the need for additional survival signals[20,21]. This could explain the observations of Reya *et al.* and Willert *et al.* as they used HSPC carrying a constitutively expressed BCL2 transgene[13,14]. In our stroma- and serum-free culture system however, the level of apoptotic cells did not respond to Wnt agonists. Moreover, induction of the BCL2 anti-apoptotic protein could not rescue the negative impact of Wnt signals on HSPC. Although the different outcomes of our studies and the ones indicating increased numbers of HSPC upon Wnt3a treatment[13,14] might still be attributed to the differences in the culture systems, the latter studies lack direct comparisons to control conditions: while HSCs cultured with Wnt3a protein were shown to repopulate recipient mice, the repopulation efficiency in the absence of Wnt3a protein was not reported.

A tripartite balance of proliferation, apoptosis and differentiation is of vital importance to maintain functional HSPC *in vivo*. Skewing the balance towards proliferation to achieve *ex vivo* expansion of functional HSPC, therefore, requires inhibition of both apoptosis and differentiation. Since our data indicates that Wnt3a has no apparent effect on the total cell proliferation and that loss of HSPC is not mediated by apoptosis, the most likely explanation would be that Wnt signals induce differentiation of the HSPC. Indeed, we observed a small increase in the number of differentiated cells in the presence of Wnt signaling agonists (Figure 2E). In line with this, we previously observed that Wnt3a protein promoted differentiation of human HSPC without affecting their proliferation or survival[19].

Investigating the role of Wnt signals on the control of HSCs *in vivo*, Luis and colleagues considered a possible role of the dosage of canonical Wnt signaling[33], showing that, while low level Wnt activation results in enhanced HSC function, high levels of Wnt activation result in impaired repopulation capacity. Moreover, two other studies report that *in vivo* constitutive activation of Wnt signaling blocks differentiation of HSPC and causes loss of repopulation capacity[15,16]. In our setting, however, with the Wnt3a concentrations we used, we did not observe a block in differentiation of HSPC, which would have led to increased numbers of HSPC. Therefore, it is unlikely that the negative effect on the number of HSPC was due to too high levels of Wnt activation in our *in vitro* culture system. Regarding enhanced repopulation capacity of HSCs in APC mutant mice with a low level of Wnt signaling activation[33], the observed effect might be due to enhanced motility or homing capacity of APC mutant HSCs rather than a result of their proliferation, which was indeed not demonstrated.

Collectively, this study shows that Wnt signaling induced by Wnt3a protein reduces the expansion of mouse LSK cells in serum-free expansion cultures by apparently promoting their differentiation without affecting their survival. Future studies may address whether additional differentiation-blocking signals may promote HSC expansion.

**Acknowledgments**

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# CHAPTER 4

# Wnt3a Protein Reduces Growth Factor-Driven Expansion of Human Hematopoietic Stem and Progenitor Cells in Serum-Free Cultures

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**Abstract**

*Ex vivo* expansion of hematopoietic stem and progenitor cells (HSPC) is a promising approach to improve insufficient engraftment after umbilical cord blood stem cell transplantation (UCB-SCT). Although culturing HSPC with hematopoietic cytokines results in robust proliferation, it is accompanied with extensive differentiation and loss of self-renewal capacity. Wnt signaling has been implicated in regulating HSPC fate decisions *in vivo* and in promoting HSPC self-renewal by inhibition of differentiation, but the effects of Wnt on the ex vivo expansion of HSPC are controversial. Here, we demonstrate that exogenous Wnt3a protein suppresses rather than promotes the expansion of UCB-derived $CD34^+$ cells in serum free expansion cultures. The reduced expansion was also observed in cultures initiated with $Lin^-CD34^+CD38^{low}CD45RA^-CD90^+$ cells, which are highly enriched in HSC and was also observed in response to activation of beta-catenin signaling by GSK3 inhibition. The presence of Wnt3a protein during the culture reduced the frequency of multilineage CFU-GEMM and the long-term repopulation ability of the expanded HSPC. These data suggest that Wnt signaling reduces expansion of human HSPC in growth factor-driven expansion cultures by promoting differentiation of HSPC.

**Introduction**

Allogeneic hematopoietic stem cell transplantation is an important part of treatment for patients suffering from hematological disorders, including leukemia, myelodysplastic syndromes, and aplastic anemia. However, many patients lack a suitable sibling or human leucocyte antigen (HLA) matched unrelated donor. Because of its rapid availability and less stringent matching criteria[1], umbilical cord blood (UCB) is an important alternative source for hematopoietic stem and progenitor cells (HSPC). However, UCB-derived HSPC significantly differ from bone marrow- and peripheral blood-derived HSPC quantitatively and qualitatively. UCB grafts contain a relatively low number of HSPC which are relatively more primitive, resulting in impaired engraftment and a delayed hematopoietic recovery[1-5], during which patients are at increased risk for severe complications, including infections and bleeding. Several approaches have been pursued to improve engraftment after UCB transplantation, including the *ex vivo* expansion of HSPC.



HSC are defined by their self-renewal capacity and the ability to generate all different hematopoietic lineages. Although *in vivo* studies demonstrated that HSPC expand after transplantation[6], robust *ex vivo* expansion of long-term repopulating HSC remains a challenge. Culturing HSPC with different combinations of hematopoietic cytokines such as stem cell factor (SCF), Fms-related tyrosine kinase 3 ligand (Flt3L), thrombopoietin (TPO) and granulocyte-macrophage colony-stimulating factor (GM–CSF) resulted in massive expansion of committed HPC which is accompanied by a loss or at best maintenance of primitive HSC with long-term repopulation ability[7-11]. Additional signals are needed to support the expansion of primitive HSC in *ex vivo* culture systems. Several novel factors, such as the immobilized Notch-ligand Delta1, copper chelator tetra-ethylenepentamine (TEPA) and signals derived from mesenchymal stromal cells, were identified that may affect self-renewal of HSC and inhibit differentiation, thereby having the potential to improve *ex vivo* expansion protocols[12-14]. In addition, numerous promising factors have been tested in a pre-clinical setting, including developmental regulators such as fibroblast growth factor signaling, insulin-like growth factor, Angiopoietin-like proteins and Pleiotrophin and chemical modulators like all-trans retinoic acid, stemregenin1 and prostaglandin E2 (reviewed by Walasek *et al*.[15]).

The Wnt/beta-catenin signaling pathway regulates cell fate decisions in many developmental processes in embryo and adult. Stimulation of cells with Wnt signaling proteins induces the stabilization and accumulation of the signal transducer protein beta-catenin, which then localizes into the nucleus where it regulates target gene expression (reviewed by Clevers *et al*.[16]). When combined with other growth factors, Wnt proteins can promote self-renewal in several types of stem cells, such as mammary, intestinal and embryonic stem cells[17-20]. Several studies, using different approaches to inhibit the Wnt signaling pathway, showed that Wnt signaling is pivotal for normal HSC function in mouse[21-23]. In addition, some reports show that treatment with recombinant Wnt3a protein or overexpression of activated beta-catenin enhances the self-renewal capacity of mouse HSC *ex vivo*[24-26]. These studies offer hope that Wnt signals may be of use in the expansion of human UCB-derived HSPC. However, other studies show that constitutive activation of beta-catenin blocks multilineage differentiation[27] and that active beta-catenin induces apoptosis in HSPC[28, 29].

In this study we investigate the effect of Wnt signals on growth factor-driven ex vivo expansion of human HSPC. We show that Wnt3a signaling reduces growth factor driven expansion of human HSPC by promoting differentiation.

## Material and Methods

### Cord blood processing, CD34+ cell selection and HSC sorting

Umbilical cord blood was collected in several hospitals using Stemcare/CB collect blood bag system (Fresenius Kabi Norge AS) containing citrate phosphate dextrose (CPD) as an anticoagulant. Approval for collection was obtained from the Medical Ethical Committee of the Erasmus University Medical Centre (MEC-2009-410) and written informed consent from the mother was obtained prior to donation of the cord blood. Within 48 hours after collection, mononuclear cells were isolated using ficoll (Lymphoprep, Fresenius Kabi Norge AS). $CD34^+$ cells were isolated with double positive immunomagnetic selection using Magnetic Activated Cell Sorting (MACS) technology according instructions of the manufacturer (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany). MACS-selected $CD34^+$ cells were either used directly in experiments or stained with anti-Lin-FITC, anti-CD38-PerCP-Cy5.5, anti-CD90-PE (all from eBioscience, Vienna, Austria), anti-CD34-PE-Cy7, anti-CD45RA-APC-H7 (both from BD Biosciences, San Jose, CA, USA) and DAPI (Sigma-Aldrich, St Louis, MO, USA) after which viable $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$-cells, highly enriched for hematopoietic stem cells (HSC)[30], were sorted using BD FACSAria Cell Sorting System (BD Biosciences, San Jose, CA, USA).

### Expansion cultures

Selected CD34+ cells and sorted $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$-cellswere cultured in serum free Glycostem Basic Growth Medium (GBGM, Glycostem, Oss, The Netherlands) or StemSpan Serum-Free Expansion Medium (SFEM, Stemcell Technologies, Grenoble, France) supplemented with 20 µg/ml low molecular weight heparin (Abbott, Wiesbaden, Germany) and the early acting growth factors SCF (50 ng/ml, Cellgenix, Freiburg, Germany), Flt3L (50 ng/ml, Cellgenix, Freiburg, Germany) and TPO (50 ng/ml, Cellgenix, Freiburg, Germany) (from now on referred to as 'SFT medium') with or without the addition of 250 ng/ml purified Wnt3a unless indicated otherwise. Cells were cultured in a volume of 1 ml in 24-well plate at a concentration of $10^5$/ml at 37 °C in 5% $CO_2$. Every 2 to 3 days, wells were split or half of the medium was refreshed. In some experiments, we used GSK3β inhibitor CH99021 (1 µM, Stemgent, Cambridge, MA, USA) as an alternative activator of the canonical Wnt pathway. Frizzled8CRD (Fr8CRD, which blocks the binding of Wnt3a to its receptor) was produced as described[31] and used at a concentration of 15 µg/ml. Wnt3a was combined in some experiments with the Aryl hydrocarbon Receptor (AhR) antagonist StemRegenin1 (SR1, 1 µM, Cellagen Technology, San Diego, CA, USA).

### Purification of Wnt3a and preparation of liposomal Wnt3a

Wnt3a-conditioned medium was collected from Drosophila S2 cells grown in suspension culture. Wnt3a was further purified using Blue Sepharose affinity and gel filtration chromatography as described[24]. Liposomes containing DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), DMPG (1,2-dimyristoyl-sn-glycero-3-phospho-rac-glycerol) (both Lipoid AG) and Cholesterol (Sigma-Aldrich, St Louis, MO, USA) at a 10:1:10 molar ratio were prepared by extrusion method. Purified Wnt3a was mixed with liposomes at a 1:7.5 ratio to achieve a total concentration of 7-10 µg/ml of Wnt3a. After mixing, the Wnt liposomes

were incubated for at least one hour on the roller coaster at 4 °C. Next, CHAPS was removed from the Wnt liposomes by dialysis at least three times in PBS 1 hour each, using dialysis membrane with molecular weight cut-off of 10 kDa at 4 °C. The Wnt liposomes were stored at 4 °C. Activity of purified Wnt3a protein and liposomal Wnt3a was determined in a luciferase reporter assay (see below).

**Luciferase reporter assay**

Mouse LSL cells, which express luciferase in response to TCF promoter binding, were routinely cultured at 37 °C and 5% CO2 in culture medium composed of DMEM (Invitrogen, Life Technologies, Bleiswijk, The Netherlands), 10% FCS, and 1% Penicillin/Streptomycin. For the activity assays, Wnt3a reagents at a concentration of 250 ng/ml were incubated in culture medium without FCS for various periods of time at 37 °C in U-bottom 96-well plates. These media were then transferred to F-bottom 96-well plates containing LSL cells, which were plated the day before at a density of 25,000 cells/well. After overnight incubation with the indicated Wnt reagents, relative luciferase units were measured with Glomax multiplate reader.



**Flowcytometry**

At serial time points in culture, absolute numbers of viable $CD34^+$ cells were determined by a single platform flowcytometric assay, using anti-CD45-FITC, anti-CD34-PE, DAPI and a calibrated number of Stem-Count Fluorospheres (all from Beckman Coulter, Fullerton, CA, USA). Within the $CD34^+$ population, the frequency of $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$-cells was determined using the antibody panel as described above for the sorting of these cells. Absolute numbers of $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$-cells were determined by multiplying the absolute number of $CD34^+$ cells obtained in the single platform analysis by the percentage of $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$-cells within the $CD34^+$ cell population. Flowcytometric analysis was performed using a BD FACSCanto (BD Biosciences, San Jose, CA, USA) and data was analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

**Transplantation of human hematopoietic cells into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice**

This study was carried out in accordance to the Dutch law on Animal Welfare and Experiments. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Erasmus University Medical Centre Rotterdam, The Netherlands. Intrabone transplantations were performed under isoflurane anesthesia. All animals were housed in groups in individually ventilated cages. Food and water were available ad libitum. NSG mice were sublethally irradiated (3 Gy) and subsequently transplanted with the progeny generated from 1,00E+05 UCB-derived $CD34^+$ cells cultured in our SFT medium, with or without the addition of 250 ng/ml Wnt3a for 7 days. Each group contained 5 mice. Engraftment was assessed every 2 weeks starting at 3 weeks after transplantation by flowcytometric analysis of the peripheral blood, using a flowcytometric panel including anti-mouseCD45-eFluor450, (eBioscience, Vienna, Austria) and anti-humanCD45-APC-Cy7 (BioLegend, London, UK). Mice were considered engrafted when human CD45 levels were

higher than 0.1%. At 17 weeks after transplantation, the mice were sacrificed by cervical dislocation and cells from femurs were analysed.

**Results**

**Wnt3a reduces growth factor-driven expansion of UCB derived CD34$^+$ cells**

To assess whether Wnt signals affect expansion of human HSPC in culture, UCB-derived CD34$^+$ cells were cultured in serum-free medium supplemented with SCF, Flt3L and TPO (SFT medium) with or without purified Wnt3a protein. After 14 days, no significant change in total nucleated cell expansion was observed in response to Wnt3a protein (Figure 1A, p=0.74). However, Wnt3a accelerated the decline in the frequency of CD34$^+$ cells that was observed during culture (Figure 1B , p<0.001 for both 7 and 14 days of culture and Figure 1C), resulting in a significantly reduced expansion of CD34$^+$ cells after 2 weeks of culture (Figure 1D). In addition, we observed a higher frequency of cells expressing lineage markers after 14 days of culture in the presence of Wnt3a protein compared with SFT medium only (Figure 1E, p<0.05). Next, we assessed the functionality of the cultured CD34$^+$ cells by performing colony forming unit (CFU) assays. The presence of Wnt3a during culture reduced the frequency of multi-lineage CFU-GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte) , while no effect was seen in the frequency of lineage committed BFU-E (Burst Forming Unit-Erythrocyte) and CFU-GM (granulocyte, macrophage) (Figure 1F). The reduction in frequency of most immature CFU and the higher frequency of lineage positive cells suggest that exogenous Wnt3a protein promotes rather than inhibits growth factor-driven differentiation of CD34$^+$ cells in expansion cultures. Next, we evaluated the dose-response relationship of Wnt signaling on the in vitro expansion of CD34$^+$ cells. At the lowest concentration of 25 ng/ml, Wnt3a had no effect on the expansion of CD34$^+$ cells relative to control SFT cultures (Figure 1G). On the other hand, the highest concentration of 2500 ng/ml Wnt3a protein resulted in a decline of total cell number (not shown) and a complete loss of CD34$^+$ cells (Figure 1G). The effect of Wnt3a on the repopulating ability of the expanded CD34$^+$ cells was assessed by transplantation of the expanded population of cells into sublethally irradiated NSG mice after 7 days of culture. All transplanted mice showed engraftment (defined as >0.1% human CD45$^+$ cells in the peripheral blood at 7 weeks after transplantation). However, the kinetics of human chimerism development in peripheral blood appeared delayed when cells were cultured in the presence of Wnt3a (Figure 1H). Lower levels of human chimerism were also observed in the bone marrow of mice 17 weeks after transplantation of cells cultured in the presence of Wnt3a (Figure 1I, 36.9% versus 10.1% respectively, p<0.05). This indicates that Wnt3a protein reduces the long-term repopulation ability of cultured CD34$^+$ cells.

**Wnt3a reduces growth factor-driven expansion of HSC**

CD34$^+$ cells constitute a heterogeneous population, including only a minor fraction of the most immature HSC subset. Wnt3a may act differentially on primitive HSC and CD34$^+$ cells with committed progenitor properties A putative differentiation-inhibiting effect of Wnt3a on HSC may be obscured by a differentiation-inducing effect on the large population of committed progenitor cells in culture. To study the effects of Wnt3a on the most immature HSC subset, we expanded Lin$^-$CD34$^+$CD38$^{low}$ CD45RA$^{low}$CD90$^+$-cells, highly enriched for

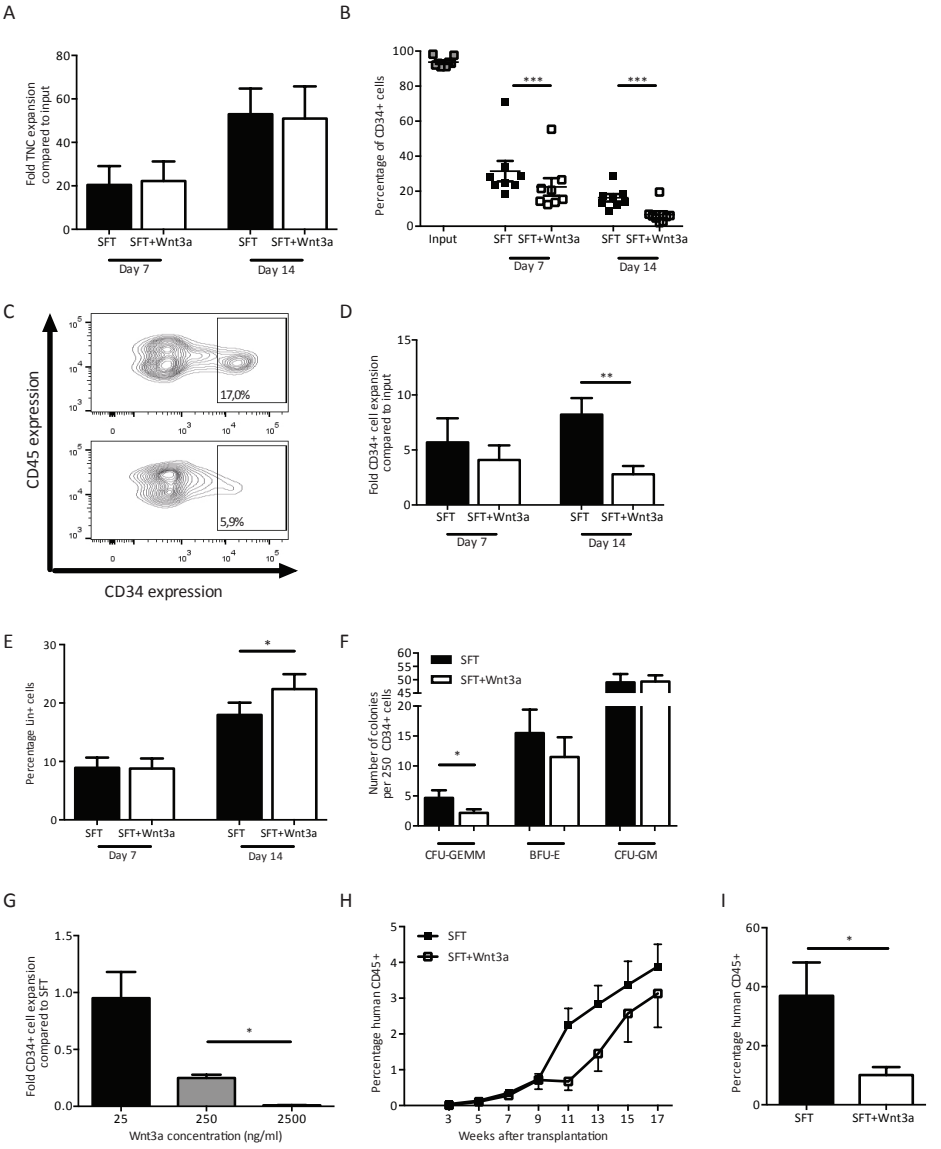


**Figure 1. Exogenous Wnt3a reduces growth factor-driven expansion of CD34⁺ cells.**
UCB-derived CD34⁺ cells were cultured in serum free SFT medium with or without the addition of Wnt3a. Cells were analyzed using flow cytometry at 7 and 14 days of culture. Shown are (A) the total nucleated cell expansion compared to input (n=8), (B) the frequency of CD34⁺ cells within the TNC population during culture (n=8), (C) expression of CD45 and CD34 after 14 days of culture in SFT with (lower panel) or without (upper panel) Wnt3a (representative of n= 8), (D) the expansion of CD34⁺ cells compared to input (n=8) and (E) the frequency of cells expressing lineage markers after 7 and 14 days of culture (n=6). (F) Frequency of CFU-GEMM, BFU-E and CFU-GM in 250 CD34⁺ cells cultured for 2 weeks in SFT or SFT+Wnt3a (n=2, 3 dishes per experiment). (G) CD34⁺ cell expansion compared to SFT medium after 14 days of culture with different dosages of Wnt3a (n=2). (H) Levels of human chimerism at several time points after transplantation with the progeny of $10^5$ CD34⁺ cells cultured for 7 days in SFT or SFT+Wnt3a medium (n=5 mice per group). (I) Levels of human chimerism in bone marrow 17 weeks after transplantation with the progeny of $10^5$ CD34⁺ cells cultured for 7 days in SFT or SFT+Wnt3a medium (n=5 mice per group). ** p<0.05, ** p<0.01, *** p<0.001*

HSC[30] in the presence or absence of Wnt3a. A similar effect of Wnt3a was observed on the expansion of the sorted Lin$^-$ CD34$^+$ CD38$^{low}$ CD45RA$^{low}$ CD90$^+$-cells. A robust total nucleated cell expansion, approximately 100-fold, was observed regardless of the presence of Wnt3a (Figure 2A). However, Wnt3a again accelerated the decline in the frequency of CD34$^+$ cells (Figure 2B, $p<0.05$ and $p<0.01$ for 7 and 14 days of culture, respectively) and led to a significantly reduced expansion of CD34$^+$ cells (Figure 2C, $p<0.01$). Moreover, Wnt3a strongly reduced the number of Lin$^-$CD34$^+$ CD38$^{low}$ CD45RA$^{low}$ CD90$^+$-cells obtained after culture, while these cells were maintained in the absence of Wnt3a (Figure 2D). These data suggest that Wnt3a inhibits the expansion of both multilineage committed progenitors and HSC.

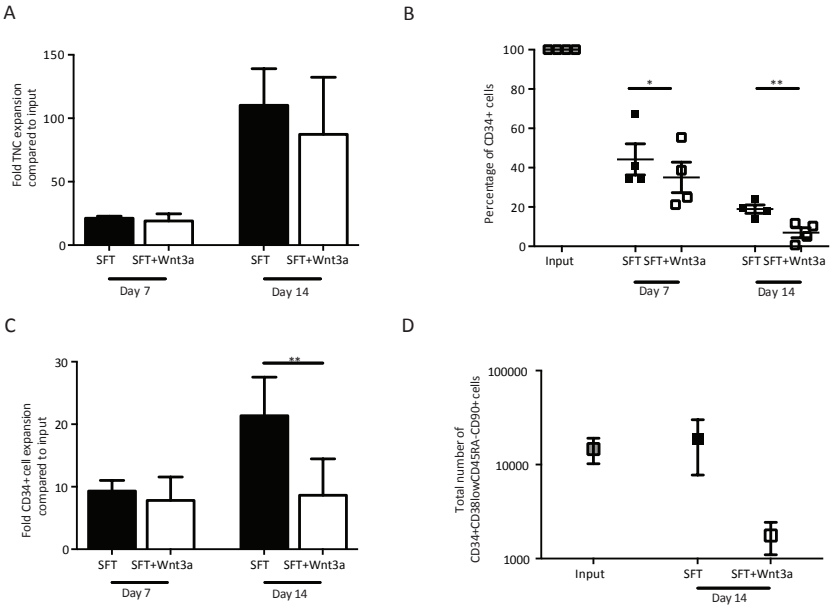


**Figure 2. Wnt3a reduces growth factor-driven expansion of HSC.**
UCB-derived DAPI$^-$Lin$^-$CD34$^+$CD38$^{low}$CD45RA$^{low}$CD90$^+$-cells were sorted out of CD34-selected cells and were cultured in SFT medium with or without Wnt3a. Flowcytometric analysis was performed at day 7 and 14. Depicted are (A) the total nucleated cell expansion in SFT and SFT+Wnt3a medium at 7 and 14 days of culture (n=4), (B) the CD34$^+$ cell frequency during culture (n=4), (C) the fold expansion of CD34$^+$ cells at day 7 and 14 of culture (n=4) and (D) the total number of DAPI$^-$Lin$^-$CD34$^+$CD38$^{low}$CD45RA$^{low}$CD90$^+$-cellsat input and after 14 days of culture in SFT or SFT+Wnt3a medium (n=4). * $p<0.05$, ** $p<0.01$

## Prolongation of Wnt3a activity does not result in increased expansion of CD34+ cells

Purified Wnt3a has been shown to have a half-life that is considerably shorter than 24 hours upon dilution in serum free media[32]. Thus, daily addition of Wnt3a protein to cell cultures would result in intermittent rather than continuous activation of the pathway. A possible explanation for our observations of reduced HSPC expansion in response to daily Wnt3a addition is that these intermittent pulses are unable to inhibit HSC differentiation. At the same time, Wnt signals may promote the differentiation of more mature CD34$^+$ cells, leading to an overall reduction of HSPC. The stability of Wnt3a protein can be increased

by association with liposomes[33, 34], and we therefore tested whether such stabilized Wnt ligands were able to prevent the decline in HSPC that we observed in response to regular Wnt3a protein. We compared the stability of purified Wnt3a protein and of Wnt3a protein associated with liposomes by incubating the proteins in serum free medium at 37°C and assessing the remaining Wnt-activity at several time points by a luciferase reporter assay. Whereas purified Wnt3a lost its activity within 8 hours, liposomal Wnt3a retained significant activity after 24 hours (Figure 3A). Despite this increased stability however, liposomal Wnt3a induced a 3.3-fold decline of CD34$^+$ cell expansion (Figure 3B), similar to purified Wnt3a. In conclusion, prolongation of Wnt3a activity does not result in increased expansion of CD34$^+$ cells.

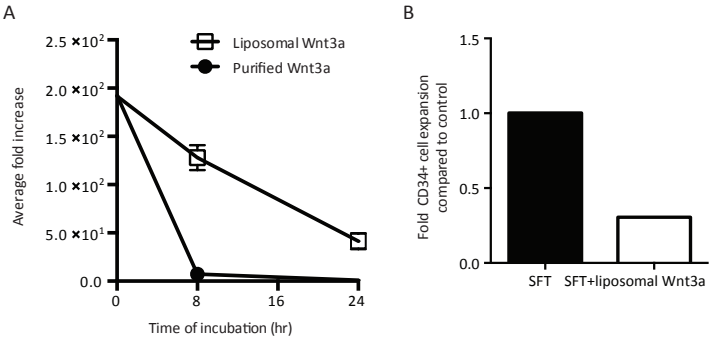


**Figure 3. Liposomal Wnt3a reduces expansion of CD34$^+$ cells.**
(A) Purified Wnt3a and liposomal Wnt3a were incubated for 0, 8, and 24 hours at 37°C in cell culture media, and transferred to LSL cells. Remaining Wnt activity was assayed by luminescence measurements. Activity plot displays average increase of luminescence over incubation time relative to background (n=10). (B) CD34$^+$ cell expansion in SFT medium with or without liposomal Wnt3a after 7 days of culture (n=1).

## Enhanced differentiation of CD34$^+$ cells is dependent on activation of the canonical Wnt pathway

Binding of Wnt3a to its receptor can be blocked with the Wnt antagonist Fz8CRD, a soluble domain of the Wnt receptor that sequesters Wnt proteins[31]. To demonstrate that the observed effects of Wnt3a were indeed dependent on binding to its receptor on HSPC, CD34$^+$ cells were cultured with or without Wnt3a and/or Fz8CRD. The negative effect of exogenous Wnt3a on the expansion of CD34$^+$ cells and the decline in frequency of CD34$^+$ cells during culture was reversed by the addition of Fz8CRD. (Figure 4A and 4B). An alternative way to activate the canonical Wnt pathway is by inhibiting glycogen synthase kinase 3β (GSK3β) which results in stabilization of the cytoplasmic β-catenin pool and subsequent transfer to the nucleus. To confirm that activation of the canonical Wnt pathway by exogenous Wnt3a underlies the inhibitory effects of Wnt3a on the expansion of CD34$^+$ cells we used a synthetic GSK3β-inhibitor (CHIR99021). Addition of CHIR99021 supressed the expansion of CD34$^+$ cells (Figure 4C) to a similar extend as purified Wnt3a protein (Figure 1D). Collectively these data show that the reduced expansion of CD34$^+$ cells is due to binding of Wnt3a protein to its receptor on HSPC and subsequent activation of canonical Wnt pathway.

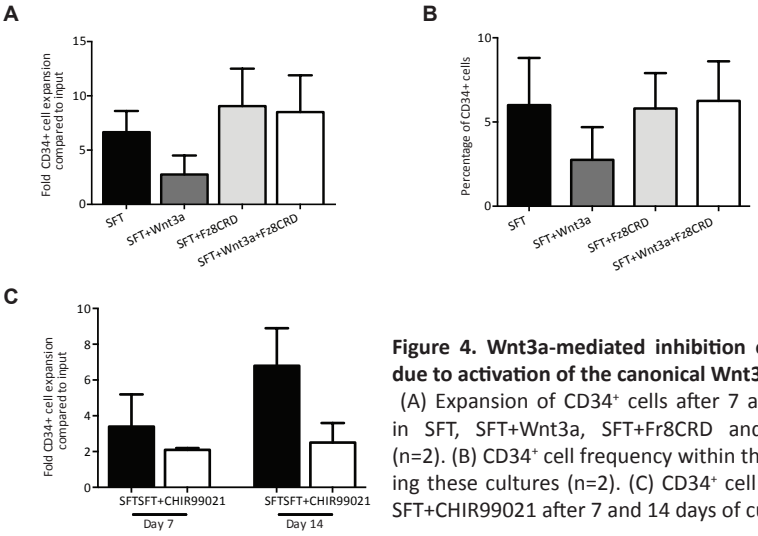


**Figure 4. Wnt3a-mediated inhibition of HSPC expansion is due to activation of the canonical Wnt3a pathway.**
(A) Expansion of CD34⁺ cells after 7 and 14 days of culture in SFT, SFT+Wnt3a, SFT+Fr8CRD and SFT+Wnt3a+Fr8CRD (n=2). (B) CD34⁺ cell frequency within the TNC population during these cultures (n=2). (C) CD34⁺ cell expansion in SFT and SFT+CHIR99021 after 7 and 14 days of culture (n=2).

## Wnt3a inhibits expansion of CD34+ cells driven by the Aryl hydrocarbon receptor antagonist Stemregenin1

The Aryl hydrocarbon Receptor (AhR) is implicated to play a role in the proliferation and differentiation of HSPC. AhR-KO mice have increased number of LSK cells in the bone marrow and these LSK are hyperproliferative[35] and hematopoietic progenitors of donor mice treated with the AhR agonist TCDD show impaired competitive engraftment[36]. In addition, the AhR antagonist StemRegenin1 (SR1) has been shown to effectively enhance the expansion of human HSPC[37, 38]. We included SR1 in our cultures as a positive control for enhancing growth factor-driven CD34+ cell expansion and in addition, to evaluate putative cooperative effects of the Wnt and AhR signalling pathways. Expectedly, SR1 indeed promoted CD34⁺ cell expansion to a similar extent as reported before[37], indicating the suitability of our SFT medium, while Wnt3a reduced CD34⁺ cell expansion (Figure 5). Addition of both Wnt3a and SR1 resulted in an increased expansion compared to Wnt alone, but a reduced expansion compared to SR1 alone (Figure 5, $p<0.01$ and $p<0.05$ respectively), showing that even in the presence of AhR pathway inhibition, Wnt3a suppresses the expansion of CD34⁺ cells.

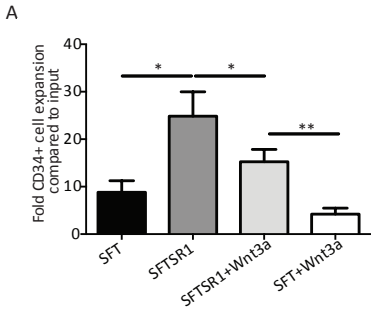


**Figure 5. Wnt3a inhibits SR1-enhanced CD34⁺ cell expansion.**
UCB-derived CD34⁺ HSPC were cultured for 14 days in SFT or SFTSR1 medium with or without the addition of Wnt3a. Flowcytometric analysis was performed at day 14. Depicted is the CD34⁺ cell expansion after 14 days of culture (n=5). ** p<0.05, ** p<0.01*

## Discussion

The effects of Wnt signaling on human HSPC proliferation, differentiation and survival are still poorly understood. In this study, we show that Wnt3a protein suppresses rather than promotes growth factor-driven expansion of UCB-derived $CD34^+$ cells in serum-free expansion cultures. Wnt3a induced accelerated differentiation of both HSC and multipotent progenitors, resulting in the production of more differentiated cells. The reduced expansion of human HSPC, appeared to be dependent on activation of the canonical Wnt signaling pathway upon binding of Wnt3a to its receptor at the cell surface.

Our findings compare well to those by Nemeth et al. , who observed a decrease in expansion of mouse LSKI (Lineage negative, Sca-$1^+$, c-$kit^+$, IL-$7R\alpha^-$) cells after culture in serum-free medium containing SCF, Flt3L and Wnt3a compared to culture conditions with growth factors only. In addition, they showed no enhanced repopulation capacity of Wnt3a-cultured LSKI cells compared to control-cultured cells[25]. Earlier, several studies showed that overexpression of beta-catenin in HSC resulted in a functional defect of hematopoiesis and loss of repopulating activity[27, 39]. These results are in line with our observations of enhanced differentiation of HSPC upon stimulation of the canonical Wnt pathway. However, Malhotra et al. reported that overexpression of activated beta-catenin may expand the pool of HSC, both phenotypically and functionally in long term cultures[40] . In addition, overexpression of the Wnt inhibitor Dickkopf-related protein 1 (Dkk1) was shown to significantly impair the self-renewal capacity of adult HSC[21, 22]. How to reconcile these contradictory results? Although at first sight contradictory, the many differences in types of cultures, cells growth factors added may explain some of the observed differences.

Willert et al. and Reya et al. earlier showed that purified Wnt3a induced proliferation, while inhibiting differentiation of HSC in growth-factor based cultures of mouse LSKT (Lineage negative, Sca-$1^+$, c-$kit^+$, Thy-$1.1^{lo}$)[24, 26]. However, they used cells derived from BCL2 transgenic mice. The BCL2 anti-apoptotic signal in HSPC may counteract possible apoptotic signals induced by Wnt3a signaling. Moreover, they used serum, which may supply additional signals that allow canonical Wnt signaling to exert a differentiation inhibitory effect or a more pronounced effect on self-renewal. It would compare well to several other studies[28, 41], which show that the presence of additional factors is required to balance the activated Wnt pathway. Trowbridge et al. showed enhanced long-term repopulation after treating mice with a GSK3beta inhibitor, which regulates the canonical Wnt pathway and several other pathways as well[41]. Perry et al. showed that the combination of a PTEN deletion and activation of beta-catenin results in enhanced self-renewal and expansion of HSC[28]. Another important pathway involved in HSC self-renewal and inhibition of differentiation is the pathway initiated by the aryl hydrocarbon Receptor (AhR). It is now well established that AhR-KO mice have increased number of LSK cells in the bone marrow and these LSK are hyperproliferative[35], while hematopoietic progenitors of donor mice treated with AhR agonist TCDD show impaired competitive engraftment[36]. In addition, the AhR antagonist StemRegenin1 (SR1) promotes expansion of human hematopoietic stem cells[37, 38]. We evaluated whether combined inhibition of the AhR pathway and activation of the Wnt pathway would result in enhanced expansion of HSPC. However, Wnt signaling also reduced the expansion of $CD34^+$ HSPC in the presence of the aryl hydrocarbon receptor antagonist SR1. Another important factor that may affect the response of HSPC to Wnt signaling is the level of oxygenation. Expansion cultures are usually performed at normoxic levels. However, it is well established that the response of HSPC to hematopoietic cytokines is different under

normoxic and hypoxic conditions, which mimics the in situ bone marrow environment[42]. The effect of stimulation of the canonical Wnt pathway on HSC expansion may be modulated by the level of oxygenation, which was already shown for the effect of the Wnt4-dependent pathway on the functional capacities of mesenchymal stem cells[43].

Apart from other cytokines and pathways involved, the dose and timing of Wnt activation might play an important role. Our data show that both intermittent pulses of Wnt3a and more continuous exposure to Wnt3a have a similar negative effect on the expansion of $CD34^+$ cells in serum-free expansion cultures. Luis et al reported that Wnts are tightly regulated in a dose-dependent fashion[44], which may affect their biological activity. Thereby, varying results (exerted via the canonical Wnt pathway) may result from different levels of Wnt signaling, such as can be achieved in different experimental conditions. The optimal level of Wnt signaling for HSC was suggested to be only slightly increased over normal physiological values, while higher levels resulted in impaired engraftment potential of HSC fashion[44].

Collectively, we show that exogenous Wnt3a proteins reduces the expansion of human HSPC in serum-free growth factor-driven HSPC expansion cultures by promoting their differentiation without apparently affecting their proliferation or survival. It cannot be excluded that additional signals, such as the induction of a hypoxic cellular response, are needed for a possible positive effect of canonical Wnt signaling on HSPC expansion. Future studies may address the fragile balance between canonical Wnt signaling and other pathways to determine whether combined activation of Wnt signaling and other signaling pathways may promote human HSPC expansion.

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# CHAPTER 5

# A liver organoid-mediated gene therapy approach for treatment of Hurler disease

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**Ongoing work**

# CHAPTER 6

# General discussion & future perspectives

With their abilities to self-renew and mature into specialized cell types of a particular tissue, adult stem cells are at the foundation of the maintenance and repair of adult tissues. Owing to their functions in restoration of lost, damaged, or aging cells and tissues in the human body, adult stem cell populations are also valuable for therapeutic applications. Today, hematopoietic stem and progenitor cells (HSPC) are routinely used in therapeutic settings[1]. Furthermore, recent advances in cultures of several other types of adult stem cells from a variety of tissues including liver and intestine opens up new experimental avenues for disease modeling, toxicology studies, regenerative medicine, and gene therapy[2]. However, several shortcomings of stem cell sources, including lack of suitable donors, low numbers of stem cells, and undefined culture conditions, limit their applicability. The ability to *ex vivo* expand adult stem cell populations in a robust and defined manner would certainly facilitate exploitation of their massive regeneration potential. Therefore, it is of vital importance to provide the cultures of adult stem cells with the essential factors that govern their cell fate decisions. Among the many pathways that are implicated in regulation of stem cell self-renewal and differentiation, the Wnt signaling cascade is distinctive due to its roles in maintenance of multiple stem cells types[3]. With the aim of developing cell culture conditions to expand stem cells, in this thesis, the effects and possible use of Wnt signaling proteins for propagation of adult stem cells from intestine, liver and blood tissue in defined culture conditions were investigated. Subsequently, we applied our advances to address a genetic disease using these stem cells.

The findings described in this thesis provide new insights into the importance of achieving stable Wnt3a activity via hydrophobic carriers for the culture of human organ stem cells from intestine and liver in serum-free conditions. Moreover, this thesis demonstrates that regardless of the stability of the Wnt3a protein, Wnt3a induced signaling leads to a reduction rather than an increase in the number of HSPC both in mice and humans. Finally, it shows preliminary data and points out the necessary steps to be taken for a liver stem cell-mediated gene therapy approach for treatment of lysosomal storage disorders (LSDs), exemplified by Hurler disease.

**Stabilizing Wnt proteins with hydrophobic carriers for their implementation in organ stem cell cultures**

The recent development of methods to expand human adult organ stem cells *in vitro* as organoids offers novel opportunities in regenerative medicine, e.g. organ stem cells may be used to restore organ function, or to correct genetic defects[4–10]. Moreover, as organoids exhibit specific functions and characteristics of the organ of origin, they are promising tools for diagnostic or drug screening purposes and might allow personalized therapy design. Efforts are under way to use organoids from cystic fibrosis and colon cancer patients to screen drugs for each individual, bringing truly personalized medicine to patients[11,12].

Human organoids require Wnt signals, provided by a serum-containing medium conditioned by a Wnt3a-producing cell line. Nevertheless, serum batches have to be screened and support only some types of organoids, complicating culture. Moreover, conditioned media introduce differentiation-inducing and undefined components that are undesirable for drug screens or clinical applications. The use of purified Wnt proteins is therefore preferred. However, attempts to replace conditioned media with purified Wnt3a protein and establish a defined culture system have been unsuccessful. In the chapter 2 of this thesis, we show that purified Wnt3a is inefficient in organoid cultures for two reasons: Wnt3a protein has a

short-half life in serum-free medium due to its hydrophobic nature, and it requires a toxic detergent, CHAPS, which is incompatible with the high concentrations of Wnt3a required to support stem cells. Here we solve both problems by exploiting Wnt's hydrophobic nature to stabilize it using lipid-modified nanoparticles instead of detergents. This both preserves Wnt activity in the absence of CHAPS, and prolongs its stability in serum-free media. We show that lipid-stabilized Wnt3a is extremely effective in supporting self-renewal of various stem cells in defined conditions, and supports the establishment of organoids from human duodenum, jejunum and liver, all in defined media.

Our work removes a major obstacle impeding the clinical application of human adult stem cells. Moreover, the prolonged activity of lipid-stabilized Wnt3a protein and the absence of CHAPS offer advantages for all tissue culture uses of Wnt3a protein. To make our technology widely available, we developed lipid-coated nanoparticles that can be added to any cell culture to instantly extend the lifetime of Wnt proteins. Moreover, as another alternative we show that Wnt3a liposomes can be freeze-dried and retain their activity upon reconstitution. Both of these alternative technologies provide efficient ways to store and distribute stabilized Wnt ligands. Highlighting the ability of lipid vesicles to replace the toxic detergent CHAPS for obtaining active Wnt proteins in our study, we also propose that readdressing the purification of Wnt proteins by involving hydrophobic carrier molecules might further advance the Wnt field.

Our findings in this chapter also demonstrate the importance of supplying the necessary signals at the adequate levels for *in vitro* expansion of adult organ stem cells. With respect to necessary signals, the organoid cultures of organ stem cells also requires a laminin and collagen-rich matrix, known as Matrigel (BD Biosiences) or Cultrex BME (Amsbio) by its trade name, which provides a scaffold for 3D growth[4–7,10,13]. These matrices consist of Englebreth-Holm-Swarm mouse sarcoma cells-derived gelatinous protein mixtures that mimic the extracellular environment in many tissues[14]. However, Matrigel or BME are not well-defined matrices and besides the extracellular components, they also contain sarcoma cell-originated growth factors. Therefore, in particular due to batch-variations, they are a source of variability, which should be avoided for reliable experimental analyses. Hence, a further step in facilitating clinical and diagnostic applications of organ stem would be the development of artificial, well-defined matrices.



**The effect of Wnt3a-induced signaling in hematopoietic stem and progenitor cells *ex vivo***

The widespread use of hematopoietic stem and progenitor cell (HSPC) transplantation in treatment of a variety of hematological and genetic diseases is limited by the shortage of compatible donors. Owing to its ease of availability and less stringent matching criteria[15], umbilical cord blood (UCB) offers advantages over other sources of HSPC. However, the use of the UCB as a source of HSPC has resulted in a delayed hematopoietic recovery and impaired engraftment in adult patients[16,17]. This has been shown to be due to the low number of HSPC in a single cord unit[18]. Therefore, strategies for development of an *ex vivo* HSC and progenitor expansion system have attracted much attention. Early attempts to increase the number of HSPC in culture by the use of cytokines have largely been unsuccessful, pointing to a need of additional factors to support HSC amplification *in vitro*. In this respect, a variety of *in vivo* loss-of-function studies indicated a requirement of canonical Wnt signaling for HSC self-renewal[19–21]. However, studies using purified Wnt3a protein for the expansion of mouse HSPC *in vitro* have generated controversial outcomes[22–24]. While Reya and Willert suggested

increased self-renewal of apoptosis-resistant, transgenic HSPC *ex vivo* upon stimulation with Wnt3a, Nemeth and colleagues reported a decrease in the number of wild type mouse HSPC cultured with Wnt3a protein[22–24]. Nevertheless, these studies either did not take into account the importance of the normal physiology or did not consider the activity of purified Wnt3a protein, which may be insufficient to promote HSPC expansion in serum-free cultures. Furthermore, the role of Wnt3a induced signaling in the expansion of human UCB-derived HSPC was not assessed. Therefore, in the chapters 3 and 4 of this thesis, the impact of Wnt3a-induced signaling in the culture of HSPC from mouse BM (Chapter 3) and human UCB (Chapter 4) was investigated.

The data presented in chapter 3 show that in a serum-free stroma-free system, purified Wnt3a-induced signaling led to a decrease in the number of mouse HSPC. Moreover, we observed a similar reduction of mouse HSPC number when we provided a more stable Wnt activity via Wnt3a liposomes. In line with our observations in mouse, we demonstrated in chapter 4 that Wnt3a-induced signaling also reduced rather than improved growth factor-driven expansion of human UCB-derived HSPC in serum-free cultures. With respect to a negative effect of Wnt signaling on the number of HSPC, several studies suggested that *in vivo* constitutive activation of Wnt signaling leads to a block in differentiation and exhaustion of HSCs[25,26]. Several other studies suggested that the decrease in the number of HSPC upon Wnt activation might result from increased apoptosis[27,28]. This could well explain the results of Reya and Willert, which indicated increased self-renewal of apoptosis-resistant HSPC upon treatment with purified Wnt3a protein[22,23]. However, in our cultures we observed neither a block in differentiation nor an increase in apoptosis. Furthermore, we found that Wnt3a did not promote self-renewal when apoptosis was inhibited using an inducible BCL2. These results argue against a role for apoptosis in the loss of HSPC in response to Wnt3a protein *in vitro*. Moreover, since no effect of Wnt3a protein on total cell proliferation in mouse HSPC cultures was observed, the decreased number of HSPC is mostly likely due to promotion of differentiation. Indeed, in chapter 4 we also demonstrated a pro-differentiation effect of Wnt signals in cultures of human UCB-derived HSPC.

Regarding the different outcomes of our studies and those implying a block of differentiation or increased apoptosis as an explanation for loss of HSPC upon Wnt activation, it is noteworthy to mention that the latter studies are based on constitutive activation of Wnt signaling *in vivo* via a constitutively active form of β-catenin[25–28], and therefore do not serve as good comparisons for an *in vitro* system where transient activation of Wnt signaling is achieved by Wnt proteins. Moreover, the *in vitro* studies of Willert and Reya, which suggest an increased self-renewal of HSPC upon Wnt3a treatment, demonstrate that HSCs cultured with Wnt3a protein repopulate the recipient mice, but not that the cells cultured without Wnt3a protein did not engraft. Therefore, these studies do not reflect a clear discrepancy to our observations.

By using different combinations of mutant alleles of a negative regulator of Wnt signaling, Apc, Luis et al generated a series of Wnt signaling levels in mice to observe the effect on HSC self-renewal[29]. There, they observed that while a low dose of Wnt signals enhances HSC function, higher levels resulted in impaired repopulation capacity. In our settings, however, different concentrations of Wnt3a proteins did not induce *ex vivo* proliferation of HSPC neither in mouse nor in human. Here, one can argue that the right dosage with the Wnt3a has not been achieved to induce HSC proliferation. Nevertheless, translating an *in vivo* constitutive activation of Wnt signaling approach to an *in vitro* culture system where activation of Wnt pathway is obtained by transient Wnt proteins to achieve expansion of

HSPC might not be feasible. Moreover, the enhanced repopulation capacity of HSCs in Apc mutant mice with low Wnt signaling dosage might not be a result of their proliferation, but their enhanced motility or homing capacity. Indeed, in their study Luis and colleagues did not demonstrate an increased number of HSCs.
Our findings in chapter 3 and 4 indicate that the goal to induce *ex vivo* amplification of HSPC will not benefit from the addition of Wnt3a proteins in the serum-free culture systems. Nevertheless, a library of other growth factors manipulating different signaling pathways, which might cooperate with Wnt signals in expansion of HSPC, can be tested.

**Liver organoid technology for a gene therapy approach of lysosomal storage disorders**

In chapter 2, we demonstrated how we brought organoid technology a step further for clinical applications, by establishing and expanding these stem cells in serum-free conditions via Wnt3a-liposomes. As another further step, in chapter 5, we investigated the utility of liver organ stem cells in development of a gene therapy approach for treatment of lysosomal storage disorders (LSDs), with initial application towards Hurler disease.
The treatments of LSDs are based on the phenomenon of 'cross-correction', which allows lysosomal enzymes secreted by one cell type to be taken up and targeted to the lysosomes of otherwise enzyme-deficient cells. The two current therapies of Hurler patients are enzyme replacement therapies (ERT) and HSC transplantations[30]. However, both treatment modalities bear significant drawbacks. Although ERT can slow the progression of some LSDs, it is very costly and rapid clearance of the enzyme from the bloodstream limits its effect[30,31]. HSC transplantation can ameliorate the symptoms of some LSDs. For example, beneficial effects of HSC transplantation are observed in Hurler's disease due to the transplanted cells secreting the missing enzyme[30–32]. However, the levels are insufficient to halt progression of the symptoms.
Several studies in mice have demonstrated that HSC transplantation could be more effective when the secretion of the missing enzyme from bone marrow HSCs is increased using gene therapy[33–35]. Nevertheless, the use of HSCs for gene therapy approaches retains the hurdles related to HSC transplantations such as shortage of suitable donors and graft-vs-host disease, early morbidity and mortality. Moreover, as HSCs cannot be *ex vivo* cultured and monitored, the oncogenic potential of genome-modifying viruses, and silencing of the transgenes, frequently observed with lentivirus, remain major concerns[36]. This underscores the importance of organoid technology by which genetically stable stem cells can be cultured over long-periods of time, clonally expanded, genetically modified, analyzed for any dangerous oncogenic genetic changes and transplanted into recipients[2]. Therefore, a gene therapy approach directed to organoids might provide high levels of the necessary enzyme safely and for the life of the patient, circumventing the drawbacks of current approaches to treatment of LSDs.
The intrinsic properties of the liver such as its well-vascularized structure and ability to secrete into the bloodstream[37] prompted us to choose liver organoids for testing the feasibility of an organ stem cell mediated gene therapy approach towards LSDs. As a model for LSDs, we picked Hurler syndrome, which is one of the most frequent LSDs and is due to an inherited deficiency of the enzyme α-L-iduronidase (IDUA)[38]. In chapter 5 of this thesis, we showed that liver organoids lacking the Idua gene were successfully derived from a mouse model of Hurler's disease and cultured over many passages. Efficient transduction of the IDUA-deficient liver organoids with lentiviral vectors expressing IDUA generated organoids, which

not only produced but also secreted high levels of IDUA enzyme over long periods of time. This establishes the first step of liver stem cell-mediated gene therapy of Hurler's disease. In the future, liver organoid clones should also be monitored to ensure the absence of any undesired genetic changes.

A second step for a liver organoid mediated gene therapy approach relies on the ability of liver organoid cells to repopulate the liver. Huch and colleagues demonstrated that liver organoid differentiate into mature hepatocytes *in vitro* and then engraft into the livers of mice upon transplantation[10,13]. For successful engraftment of liver organoid derived cells into liver parenchyma, it is necessary to open up stem cell niches. In our study we adapted the acute damage model, which was previously shown to generate a permissive environment allowing normal and liver organoid-derived hepatocytes engraftment. For this, recipient mice were pre-treated with the hepatotoxic reagent $CCl_4$, inducing partial ablation of hepatocytes[39,40] and with the alkaloid, monocrotaline, which inhibit the proliferation of the remaining hepatocytes[39,41]. Indeed, increased serum transaminase levels and damaged liver morphology verified the hepatotoxicity of these reagents. Nevertheless, in chapter 5 we showed that although liver organoid derived cells reached to the livers of the recipient mice via intrasplenic injection technique, these cells did not engraft into recipient mice. Since achieving successful engraftment of liver organoids is essential for our strategy of liver organoid mediated gene therapy of Hurler disease, the investigation of probable reasons behind inefficient transplantations is necessary. Therefore, further experiments are required to assess whether the damage model we used in this study indeed formed a regenerative environment that allows the engraftment of liver cells. Transplantations of freshly isolated, genetically marked hepatocytes into pre-treated recipients might aid in testing the efficiency of the indicated conditioning regimens and might serve as a positive control. Accordingly, the doses of the toxic agents can be adjusted. Guo and colleagues report that $CCl_4$ treatment following transplantation increase the efficiency of hepatocyte engraftment[39]. This strategy was based on the fact that the intrasplenically injected cells deposit in periportal locations while $CCl_4$ would only affect the perivenous hepatocytes[42]. The $CCl_4$ treatment after transplantations would thus induce a further proliferation stimulus for the transplanted cells in non-ablated regions[42]. Therefore, in future studies this regimen might be tested to see whether it increases the efficiency of liver organoid derived cells engraftment into the liver.

Exploring the probable reasons, we observed that the differentiation protocol we used did not lead to efficient specification of liver organoid cells into mature hepatocytes. Thus, further investigations of pathways known to influence differentiation of liver organoids towards hepatocytes, including Wnt, TGFβ, BMP, cAMP and Notch signaling pathways[13], might enhance the engraftment potential of liver organoids. The low differentiation efficiency might also stem from the presence of low numbers of Lgr5+ stem and progenitor cells in liver organoids, which are routinely cultured with R-spondin but not with Wnt3a proteins. Our data showed that by activation of Wnt signaling via liposome-stabilized Wnt proteins, the population of Lgr5+ GFP expressing organoid cells was drastically increased. This increase in the number of bi-potent Lgr5+ stem and progenitor cells might increase the yields of mature hepatocytes. A further optimization step that can be explored in case of insufficient engraftment is the additional use of mesenchymal stromal cells shown to support regeneration, by blood vessel formation[43] or by suppressing the immune system[44].

To sum up, in this chapter we demonstrated the utility of liver organoids as a stem cell source for production of the enzymes necessary for treatment of LSDs, in particular Hurler

disease. A major obstacle is now to obtain sufficient numbers of mature hepatocytes with robust engraftment potential from the organoids. Once this obstacle is overcome, the liver organoid based therapies will offer a safe source of liver cells for treatment of a variety of diseases.

**Concluding remarks**

Adult stem cells cells are capable of regenerating and repairing damaged tissues, and therefore offer scientists the possibility of creating new therapies and cures for a wide variety of diseases. In this thesis, we conducted studies aiming at investigating the use of Wnt proteins for *in vitro* culture of adult stem cells. Our innovative liposome-stabilized Wnt technology now enables defined culture of adult organ stem cells, allowing clinical applications mediated by these stem cells.
Our studies establishing a negative effect of Wnt proteins in the expansion of adult HSPC *in vitro* also underscores differential regulation of different stem cell types by Wnt signaling. Future studies might be necessary to answer whether the origin of the stem cells is an important determinant for the effect of Wnt signals.
Applying the organ stem cell technology for treatment of LSDs, we expect to overcome the major obstacle holding back their gene therapy: the delivery of genes to the appropriate cells without unwanted side effects like genotoxicity, or targeting the wrong cells, or provoking the immune system with viruses. Establishing such a novel and safe application of gene therapy in mouse models, would open up new avenues for treatments of many other diseases.

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# Addendum

**Summary**

The maintenance of homeostasis and repair in tissues and organs throughout adult life relies on stem cells of the respective tissues, such as blood, intestine or liver. These stem cells could be of great value for clinical purposes because of their capability to regenerate lost or damaged tissues. For reasons such as low availability of stem cells, lack of competent donors and difficulties in expanding the cells, the development and application of stem cell-based therapies has so far been limited. Recent progress in the identification, isolation and expansion of adult stem cells promises to advance their clinical use. The identification of the factors necessary for propagation of the stem cells is an essential element in furthering this strategy. Among the many cell signaling pathways indicated in control of stem cell self-renewal and differentiation, the Wnt signaling pathway stands out due to its roles in maintenance of stem cells from a variety of tissues. In this thesis we investigate the effects and applicability of Wnt signaling proteins in propagation of adult stem cells from intestine, liver and blood, with the goal of improving their use for clinical purposes.

The establishment of human organ stem cell cultures as organoids has opened new avenues for stem cell-based assays and therapies. The human organoids are dependent on Wnt signals provided in the form of a Wnt3a-conditioned medium. While effective, this medium contains serum and other undefined factors undesirable for clinical and diagnostic applications. In **Chapter 2**, we investigate the use of purified Wnt3a protein instead of a Wnt3a-conditioned medium. We find however that purified Wnt3a protein fails to support human intestinal stem cells. We show that this is because the Wnt3a rapidly loses its activity upon dilution in serum-free medium. In addition, purified Wnt3a contains a detergent, CHAPS, that is required to maintain the stability of Wnt3a but is toxic to the stem cells. Searching for an alternative way to stabilize Wnt3a protein, we explored the finding that Wnt3a protein can associate with liposomes, small aggregates of fatty molecules. We show that these liposomes can replace CHAPS and maintain Wnt3a activity in the absence of serum or detergent. Importantly, the lipid-stabilized Wnt3a supports both serum-free establishment and expansion of organoids from healthy and diseased human organs, including intestine and liver. These findings remove a major impediment for clinical and diagnostic applications of adult stem cells, and offers advantages for all cell culture uses of WNT3A protein. **Chapters 3 and 4** of this thesis describe the effect of Wnt3a protein in *ex vivo* culture of hematopoietic stem and progenitor cells (HSPC). In **Chapter 3,** we show that Wnt3a protein leads to a reduction in the number of mouse HSPC in our stroma-free, serum-free culture system. Our data demonstrate that increased stability of Wnt activity by lipid-associated Wnt3a proteins also negatively affects the number of mouse HSPC. Investigating a possible reason for Wnt-induced loss of these HSPC, we found no evidence for apoptosis, but a tendency for increased differentiation of HSPC. In agreement with the mouse study, in **Chapter 4**, we show that Wnt3a protein decreases the number of human umbilical cord blood-derived HSPC in serum-free expansion cultures due to increased differentiation. Taken together, our data show that *ex vivo* propagation of HSPC may require additional differentiation-inhibiting factors.

In **Chapter 5**, we address the use of liver stem cells cultured in organoids for gene therapy of lysosomal storage disorders, with initial application towards Hurler disease. Hurler disease is caused by a deficiency of the lysosomal enzyme α-L-Iduronidase, which leads to accumulation of its undegraded substrates and a variety of subsequent clinical symptoms in a diversity of tissue systems. The treatment of Hurler disease is based on exogenous

administration of the missing enzyme to patients. In this regard, stem cells, which can self-renew, differentiate and reconstitute target organs, might function as the permanent donors of the normal enzyme. Moreover, for providing a high level functional enzyme to the patients, stem cells should be genetically modified to overexpress the missing protein. Therefore, *ex vivo* gene therapy of stem cells to provide high levels of the missing enzyme for a lifetime to the patients of Hurler disease, or most other LSDs, is an attractive therapeutic modality. Here, we show that liver organoids can be derived from the mouse model of Hurler disease and cultured over long periods of time. Moreover, we demonstrate that genetically modified liver organoids efficiently produce and secrete the missing enzyme over many passages. Nevertheless, sufficient engraftment of the modified liver organoids for correction of the disease phenotype is yet to be established and might necessitate increased differentiation efficiency towards hepatocytes. Setting up such successful liver organoid transplantations might enable liver organoid-based therapies for treatment of a variety of genetic and liver-related diseases.

Finally, the significance and implications of the findings described in this thesis are integrated in **Chapter 6**, where also perspectives for further studies are provided. With respect to improving adult stem cell-based therapies, defining how or if we could use signaling molecules *in vitro* for expansion of stem cells are of great importance. In this regard, this thesis provides novel insights into the effects and applicability of Wnt signaling proteins in serum-free culture of adult stem cells. Further studies will address how to exploit the potential of adult stem cells and translate the basic knowledge into clinical applications.

**Nederlandse samenvatting**

Het handhaven van homeostase en herstel van de weefsels en organen in ons lichaam is de taak van stamcellen die in weefsels zoals bloed, darmen en de lever aanwezig zijn. Deze stamcellen kunnen van grote waarde zijn voor klinische doeleinden vanwege hun vermogen om verloren of beschadigde weefsels te regenereren. De ontwikkeling van stamcel-gebaseerde therapieën is echter beperkt vanwege redenen zoals het ontbreken van voldoende donoren, de beperkte beschikbaarheid van stamcellen en problemen met het vermeerderen van de cellen in celkweek. Recente vooruitgang in het identificeren, isoleren en vermeerderen van adulte stamcellen biedt nieuwe beloften voor klinische toepassingen van deze cellen. Een belangrijk element om dit mogelijk te maken is de identificatie van de factoren die vermeerdering van stamcellen in celkweek mogelijk maken. Onder de vele cellulaire signaal paden die de zelfvernieuwing en differentiatie van stamcellen reguleren, onderscheidt de Wnt-signaleringsroute zich door de regulering van stamcellen in een veelvoud aan weefsels. In dit proefschrift onderzoeken we de effecten en toepassingen van Wnt signaal eiwitten bij het manipuleren van adulte stamcellen van darm, lever en bloed, met als doel het gebruik van deze stamcellen voor klinische doeleinden te verbeteren.
Het ontwikkelen van kweeksystemen voor humane stamcellen in de vorm van zogenaamde organoiden heeft nieuwe perspectieven geopend voor de ontwikkeling van stamcel-gebaseerde testen en therapieën. Humane organoiden zijn afhankelijk van Wnt signalen en deze worden toegevoegd in de vorm van een Wnt3a-geconditioneerd medium. Dit medium bevat echter ook serum en andere ongedefinieerde factoren die ongewenst zijn in klinische en diagnostische toepassingen. Derhalve hebben we in **hoofdstuk 2** het gebruik van gezuiverd Wnt3a eiwit in plaats van Wnt3a-geconditioneerd medium bestudeerd. We vinden echter dat gezuiverd Wnt3a eiwit de kweek van humane darmstamcellen niet ondersteunt. We laten zien dat dit komt doordat het Wnt3a snel zijn activiteit verliest wanneer het verdund wordt in serumvrij medium. Bovendien bevat het een detergens, CHAPS, dat nodig is voor de stabiliteit van het Wnt3a maar wat toxisch is voor de stamcellen. In dit hoofdstuk laten we vervolgens zien dat Wnt3a eiwit bindt met liposomen, kleine aggregaties van vetachtige moleculen. Deze liposomen nemen de plaats in van het CHAPS en zorgen voor het behoud van Wnt3a activiteit in de afwezigheid van serum en CHAPS. Tenslotte laten we zien dat het liposoom-gestabiliseerde Wnt3a het serumvrij kweken en expanderen van organoiden van gezonde en zieke humane organen als de darm en lever mogelijk maakt. Deze bevindingen ruimen een belangrijk obstakel uit de weg dat klinische toepassingen van stamcellen bemoeilijkt.
**Hoofdstuk 3 en 4** van dit proefschrift beschrijven het effect van Wnt3a eiwit bij het kweken van stamcellen van het bloedsysteem (HSPC: hematopoietic stem and progenitor cells). In **hoofdstuk 3** laten we zien dat Wnt3a eiwit de vermeerdering van muizen HSPC in een stroma-vrij, serumvrij kweeksysteem tegenwerkt. Ook wanneer de stabiliteit van het Wnt3a eiwit verhoogd wordt door associatie met liposomen leidt het tot verlies van HSPC in plaats van vermeerdering. We vonden geen aanwijzingen dat Wnt3a celdood induceert in HSPC, maar juist differentiatie van de cellen bevordert. In overeenstemming met de muizenstudie laten we in **hoofdstuk 4** zien dat Wnt3a ook de differentiatie en verlies van HSPC uit humaan navelstrengbloed veroorzaakt. Deze observaties suggereren dat voor vermeerdering van HSPC in celkweek het nodig zal zijn om aanvullende differentiatieblokkerende signalen te identificeren.

In **hoofdstuk 5** onderzoeken we of lever stamcellen, gekweekt als organoiden, kunnen worden ingezet voor een vorm van gentherapie, in eerste instantie toegepast voor de ziekte van Hurler, een voorbeeld van een lysosomale stapelingsziekte. Deze ziekte wordt veroorzaakt door een gebrek aan het lysosomale enzym α-L-Iduronidase, wat leidt tot accumulatie van cellulaire afbraakproducten en ernstige klinische symptomen. Toediening van het enzym kan de symptomen verminderen. We onderzoeken daarom of genetische modificatie van lever stamcellen, zodat deze het enzym uitscheiden, een oplossing kan bieden. We laten zien dat we lever organoiden kunnen verkrijgen van een muizenmodel van de ziekte van Hurler en deze voor langere tijd in kweek kunnen houden. Na genetische modificatie zijn de lever organoiden effectief in het produceren en uitscheiden van het ontbrekende α-L-Iduronidase enzym, zelfs na vele passages. De volgende uitdaging is het effectief transplanteren van de gemodificeerde cellen in de patient om voldoende enzym in de bloedbaan te krijgen voor een succesvolle behandeling van de symptomen. Hiervoor zal het wellicht nodig zijn om verbeterde differentiatie protocollen te ontwikkelen om uitgerijpte levercellen van de organoiden te verkrijgen. Dit zou lever organoide-gebaseerde therapieën mogelijk maken voor verschillende genetische en lever-gerelateerde ziekten.
Het belang en mogelijke implicaties van de bevindingen beschreven in dit proefschrift worden tot slot beschreven in **hoofdstuk 6**. Daarnaast worden in dit hoofdstuk ook adviezen voor verdere studies gedaan. Met betrekking tot het verbeteren van adulte stamcel-gebaseerde therapieën, is het van groot belang te bepalen hoe en of we signalerings moleculen *in vitro* kunnen gebruiken voor de expansie van stamcellen. In dit opzicht biedt dit proefschrift nieuwe inzichten in de effecten en toepasbaarheid van Wnt signaal eiwitten in het serumvrij kweken van adulte stamcellen. Verdere studies zullen nader ingaan op het benutten van de potentie van adulte stamcellen, en hoe fundamentele kennis vertaald kan worden naar klinische toepassingen.

***Curriculum vitae***

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**Education**

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**Publication list**

1. **Tüysüz N**, van Bloois L, van den Brink S, Begthel H, Verstegen MM, Cruz LJ, van der Laan LJ, de Jonge J, Vries R, Braakman E, Mastrobattista E, Cornelissen JJ, Clevers H, ten Berge D. **2015**. Serum-free culture of human organ stem cells by lipid-mediated stabilization of Wnt ligands. **(Submitted)**
2. **Tüysüz N,** Duinhouwer LE, Kurek D, Rombouts EW, van Bloois L, Mastrobattista E, Cornelissen JJ, Braakman E, ten Berge D. **2015**. Wnt3a protein reduces expansion of mouse hematopoietic stem and progenitor cells in serum-free cultures. **(Submitted)**
3. Duinhouwer LE, **Tüysüz N**, Rombouts EW, ter Borg MN, Spanholtz J, Cornelissen JJ, ten Berge D, Braakman E. **2015**. Wnt3a protein reduces growth factor-driven expansion of human hematopoietic stem and progenitor cells in serum-free cultures. **(*PLos One*)**
4. Narcisi R, Cleary MA, Brama P, Hoogduijn MJ, **Tüysüz N**, ten Berge D, van Osch G. **2015.** Long term expansion, enhanced chondrogenic potential and suppression of endochondral ossification in-vivo of adult human MSCs via modulation of WNT signalling. (***Stem Cell Reports***)
5. Kurek D, Neagu A, Tastemel M, **Tüysüz N**, Lehmann J, van de Werken HJ, Philipsen S, van der Linden R, Maas A, van IJcken W, Drukker M, ten Berge D. **2015.** Endogenous WNT Signals Mediate BMP-Induced and Spontaneous Differentiation of Epiblast Stem Cells and Human Embryonic Stem Cells. (***Stem Cell Reports***)
6. Yin Y, Bijvelds M, Dang W, Xu L, van der Eijk AA, Knipping K, **Tüysüz N**, Dekkers JF, Wang Y, de Jonge J, Sprengers D, van der Laan LJ, Beekman JM, Ten Berge D, Metselaar HJ, de Jonge H, Koopmans MP, Peppelenbosch MP, Pan Q. **2015.** Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. (***Antiviral Research*)**
7. Gültas M, Haubrock M, **Tüysüz N**, Waack S. **2012.** Coupled mutation finder: a new entropy-based method quantifying phylogenetic noise for the detection of compensatory mutations. (***BMC Bioinformatics***)

**Ph.D Portfolio**

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2011 Genetics (G) course
2011 Biochemistry and biophysics (BB) course
2012 Cell and Developmental Biology (CDB) course
2012 Safely working in the laboratory
2012 Literature training course

***Specific courses***

2011 Laboratory animal science (Art. 9)
2011 Transgenesis, gene Targeting and in vivo imaging
2012 In vivo Imaging: from molecule to organism

***Workshops***

2011 4th Innovative Mouse Model workshop, Leiden, the Netherlands

2011 17th MGC workshop, Maastricht, the Netherlands

2012 18th MGC workshop Düsseldorf, Germany *(poster presentation)*

***Conferences***

2012 International Society of Differentiation Conference, 'Stem cells, Development and Regulation', Amsterdam, the Netherlands (Poster presentation)

2012 40th Annual meeting of the International Society of Experimental Hematology, Amsterdam, the Netherlands (Poster presentation)

2013 Dutch Society for Stem Cell Research (DSSCR), Utrecht, the Netherlands

2013 Wnt symposium, Heidelberg, Germany (Poster presentation)

2014 Netherlands Society of Gene and Cell Therapy (NVGCT) symposium, Lunteren, the Netherlands (Poster presentation)

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***Teaching/ Supervision***

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2014 Supervision of master student (Tania M. Garcia)

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