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„Identification and characterization of novel regulators in hematopoietic stem cells“

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To my parents

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Abstract

Hematopoietic stem cells (HSCs) are doubtless one of the best functionally characterized tissue stem cell types in the human body. Decades of research and development in the area of stem cell biology led to great advances in the understanding of the mechanisms controlling hematopoietic stem cell functions. The incredible regenerative potential of HSCs has paved the way for their therapeutic use to treat a broad range of diseases. However, despite the extensive research in this field, the mechanisms that govern HSC stemness remain poorly understood. This fact unfortunately restricts the development of strategies that aim to expand HSCs for clinical benefits. *Ex vivo* expansion of transplantable stem cells is an especially promising strategy for umbilical cord blood (UCB)-derived HSCs, which could be utilized as an alternative source to bone marrow (BM)-derived stem cells. However, the low number of HSCs in a single cord blood unit stands out as the major obstacle for the use of UCB for allogeneic transplantations.

The aim of this thesis was to discover novel genetic regulators of UCB-HSC expansion and to explore the molecular mechanisms, which are involved in cell regulation after silencing of the selected candidates. For identification of novel modulators of UCB-derived HSCs, selected candidate genes were silenced using a lentiviral-mediated RNAi approach, followed by the assessment of the gene silencing effects in long-term cultures of HSCs. Candidate genes, which exhibited an enhanced proliferative potential combined with the maintenance of a primitive phenotype, were subjected for gene expression analysis, followed by assays that aimed to understand the molecular mechanisms, which drive stem cell expansion.

Among the selected candidates, we identified tRNA splicing endonuclease subunit 15 (TSEN15) as a potential negative regulator of HSC propagation *in vitro*. TSEN15 is part of the human tRNA-splicing endonuclease (TSEN) complex, which catalyzes the excision of introns from precursor-tRNA molecules. Knockdown of TSEN15 by shRNAs in primary human UCB $CD34^+$ cells resulted in an increased cell expansion in culture and an accumulation of primitive $CD34^+CD90^+$ cells compared to the control. Beyond the effects on stem cell expansion, we also evaluated the multipotent character of HSCs to differentiate into mature blood cells upon TSEN15 inhibition. We could observe that knockdown of TSEN15 did not affect the overall clonogenic capacity of the cells. However, TSEN15-targeted cells exhibited a reduced CD71 expression on erythroid progenitors, while expression of the myeloid markers CD11b and CD15 was elevated, thus indicating that TSEN15 knockdown may cause a skewing towards myeloid cell production. Even though further investigations are required, TSEN15 can be considered a novel potential modulator of human UCB-HSC propagation *ex vivo*.

Zusammenfassung

Hämatopoetische Stammzellen (HSZ) gehören zweifellos zu den am besten charakterisierten Gewebestammzelltypen im menschlichen Körper. Jahrzehntelange Forschung und Entwicklung auf dem Gebiet der Stammzellbiologie führten zu Fortschritten im Verständnis der Mechanismen, die die Funktionen der HSZ steuern. Das regenerative Potenzial von HSZ hat den Weg für ihren Einsatz zur Behandlung von vielen Krankheiten geebnet. Jedoch bleiben trotz der umfangreichen Forschung auf diesem Gebiet die Mechanismen, die die Selbsterneuerung der HSZ steuern, noch weitgehend ungeklärt. Diese Tatsache schränkt die Entwicklung von Strategien ein, die darauf abzielen, HSZ für den klinischen Nutzen *in vitro* zu expandieren. Die *ex vivo* Expansion transplantierbarer Stammzellen ist eine vielversprechende Strategie für HSZ, die aus Nabelschnurblut (NSB) gewonnen werden, da es sich dabei um eine wertvolle Quelle für Stammzellen handelt. Die geringe Anzahl von HSZ in einer NSB-Konserve stellt jedoch das größte Hindernis für die Verwendung dieser HSZ für allogene Transplantationen dar.

Das Ziel dieser Arbeit war es, neue genetische Regulatoren von NSB-HSZ für die *ex vivo* Expansion zu identifizieren und die molekularen Mechanismen, die an der Zellregulation nach der Stilllegung der ausgewählten Kandidatengene beteiligt sind, zu erforschen. Zur Identifizierung neuer Modulatoren von NSB-HSZ wurden ausgewählte Kandidatengene mithilfe eines lentiviral-vermittelten RNAi-Ansatzes stummgeschaltet. Anschließend wurden die Gen-Silencing-Effekte in Langzeitkulturen bewertet. Kandidatengene, die ein erhöhtes proliferatives Potenzial in Kombination mit der Aufrechterhaltung eines primitiven Phänotyps aufwiesen, wurden einer Genexpressionsanalyse unterzogen. Dies war gefolgt von Assays, die darauf abzielten, die molekularen Mechanismen zu verstehen, die die Stammzellexpansion vorantreiben.

Unter den ausgewählten Kandidaten identifizierten wir TSEN15 als einen potenziellen negativen Regulator der HSZ-Vermehrung. TSEN15 ist ein Teil des TSEN-Komplexes, der die Exzision von Introns aus Vorläufer-tRNA-Molekülen katalysiert. Die Stilllegung von TSEN15 mithilfe von shRNAs in humanen NSB-$CD34^+$ Zellen führte zu einer erhöhten Zellexpansion und einer Akkumulation von primitiven $CD34^+CD90^+$ Zellen. Neben den Auswirkungen auf die Stammzellexpansion haben wir auch den multipotenten Charakter von HSZ nach der Stilllegung von TSEN15 untersucht. Wir konnten beobachten, dass die Stilllegung von TSEN15 die klonogene Kapazität der Zellen nicht beeinflusst hat. Allerdings zeigten diese Zellen eine verminderte CD71-Expression auf erythroiden Vorläuferzellen, während die Expression der myeloiden Marker CD11b und CD15 erhöht war, was darauf hindeutet, dass die

Stilllegung von TSEN15 eine Verzerrung in Richtung der myeloiden Zellproduktion verursachen kann. Auch wenn weitere Untersuchungen erforderlich sind, kann TSEN15 als ein neuer potenzieller Regulator der *ex vivo* Expansion von NSB-HSZ betrachtet werden.

1. Introduction

Blood is an essential fluid tissue for maintaining the life of an individual. It is composed of multiple cell types that have distinct functions within a human body. Each circulating cell in the blood stream has a relatively short lifespan and undergoes continues turnover. The adequate production of blood cell populations is ensured by a process referred to as hematopoiesis. Hematopoiesis describes a tightly regulated and dynamic process of blood cell formation from a very small population of cells called hematopoietic stem cells. These rare cells are characterized by the capacity for self-renewal and differentiation into all mature blood cells. Their fascinating ability to replenish all cells of the hematopoietic lineage, at the same time maintaining a pool of HSCs throughout the life has been a subject of extensive studies for decades with still many questions remaining elusive (Seita and Weissman, 2010). The introduction will give an overview about HSCs, their niche, and their use for clinical applications, particularly stem cell transplantations.

1.1. Stem cells – general overview

Nowadays, the term 'stem cells' is mainly brought in connection with the therapeutic potential of these special cells. This is due to their main hallmark of stemness, which is defined as the capacity for perpetual self-renewal and continuous differentiation into specialized cell types (Melton, 2014). The increasing excitement about stem cells and their use in regenerative medicine led to growing interest in stem cell research and the popularity of stem cell-based treatments (Lukovic *et al.*, 2014). Stem cell research covers a broad area since stem cells can be derived from 2 main origins: embryonic and adult body tissues, giving rise to embryonic stem cells (ESCs) (Fig.1a) and adult stem cells (ASCs) (Fig.1b) (Alvarez *et al.*, 2012). In 2006, the field of stem cells has been revolutionized by the groundbreaking discovery of induced pluripotent stem cells (iPSCs), artificially reprogrammed cells with similar properties to ESCs (Fig.1c). Shinya Yamanaka and his team succeeded in the development of pluripotent cells from mouse fibroblasts by inducing the overexpression of 4 transcription factors: Oct3/4, Sox2, Klf4, and c-Myc (OSKM) (Takahashi and Yamanaka, 2006). One year later, Yamanaka's lab presented a similar reprogramming approach for the generation of iPSCs from human fibroblast (Takahashi *et al.*, 2007). These findings enabled to overcome the ethical concerns associated with the use of ESCs and set a milestone in the history of stem cell research (Omole and Fakoya, 2018).

The two naturally occurring stem cell types in mammals, ESCs and ASCs, differ considerably from each other. Firstly, ESCs are derived from the inner cell mass (ICM) of a pre-implantation blastocyst and are limited by their temporal existence during embryonic development. In contrast, ASCs, also known as somatic or tissue-specific stem cells, are derivatives of ESCs in the postnatal organism and reside throughout life in a specific microenvironment, also known as "stem cell niche" (Alvarez *et al.*, 2012). Aside from their presence at different stages of life, another key difference between those two stem cell types is their degree of "potency". ESCs are pluripotent cells, which are characterized by an almost indefinite proliferation potential and the ability to differentiate into all cell types of the body. In contrast, ASCs, an example for multipotent stem cells, have a more limited differentiation profile and can give rise to distinct somatic cell types of one particular lineage (mesoderm, endoderm, ectoderm) (Berdasco and Esteller, 2011; Abou-Saleh *et al.*, 2018).

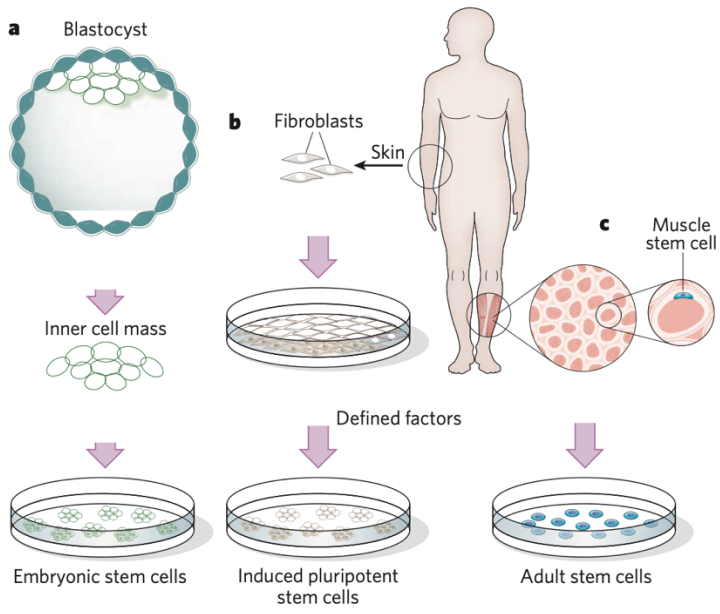


Figure 1. Stem cell types. | **a.** Pluripotent embryonic stem cells are derived from the inner cell mass of a blastocyst during early fetal development and possess the capability to differentiate into cells of all three germ layers. | **b.** Human fibroblasts that have been reprogrammed to induced pluripotent stem cells using various reprogramming techniques, share similar characteristics with their human ESC (hESC) counterparts. | **c.** Tissue-specific stem cells, such as muscle stem cells, are defined by their multipotency and play a key role in maintaining the homeostasis of mature body tissues (e.g. bone marrow, adipose tissue, lung, heart) (adapted from Lutolf *et al.*, 2009).

The class of adult stem cells resides in differentiated tissues and ensures life-long tissue homeostasis and regeneration in case of injuries and diseases. This small reservoir of stem cells comprises less than 1 % of total cells in adult tissues and is localized in specific *in vivo* microenvironments, or niches. ASCs reside in their niches in a quiescent state and divide infrequently to self-renew or give rise to tissue-specific progenitor cell types (Bhartiya, 2021). Generally, a stem cell niche must be considered as a dynamic and heterogenous microenvironment, at which intrinsic and extrinsic signals as well as the surrounding cells shape stem cell properties and define their potential. Aside from that, stem cell niches serve as protective locations that govern stem cell quiescence and activation and influence stem cell behavior. However, due to the existence of multiple somatic stem cell niches there are considerable differences in the anatomy as well as regulatory pathways. Also, the knowledge remains limited, despite extensive research, and many questions remain still unanswered, such as for instance when and how most somatic stem cell niches develop (Fuchs, Tumbar and Guasch, 2004; Ohlstein *et al.*, 2004; Scadden, 2006). The most extensively studied somatic stem cell niche today is that of the hematopoietic system.

1.2. The hematopoietic system

1.2.1. Cells of the hematopoietic system

The hematopoietic system forms a complex network with a plethora of intertwined interactions between many cellular components and organs. This system consists of more than 10 different blood cell types, which differ in their morphology and function. The 3 major cell types are the erythrocytes (red blood cells; RBCs), leukocytes (white blood cells; WBCs) and thrombocytes (platelets). Erythrocytes are the most abundant cells with 2.5×10^{13} total circulating RBCs in an adult human weighing ~70 kg (Franco, 2012). Aside from that, they are probably one of the most intriguing cell types of the human body. Unlike most eukaryotic cells, RBCs do not have nuclei and lack organelles, such as the Golgi apparatus, endoplasmic reticulum, and mitochondria. After the erythroid maturation process, when the nucleus is expelled and organelles are eliminated, RBCs take on the characteristic shape of a biconcave disk (Moras, Lefevre and Ostuni, 2017; Palis, 2014). It is assumed that this specific shape in connection with a flexible membrane, which facilitates elastic deformation of the cell when passing through small capillaries, enables RBCs to transport oxygen and carbon dioxide between the body's tissues more efficiently (Diez-Silva *et al.*, 2010).

Leukocytes are important components of the blood cell system and key players in the host's immunity against infectious organisms. In contrast to erythrocytes and thrombocytes, the group of leukocytes is very heterogenous and can be further classified into different cell types. The mammalian immune system is typically divided into the innate and adaptive immune response. While the innate immunity is the first and antigen-unspecific response to intruding pathogens, the adaptive immunity is characterized by its specificity and long-lived immunological memory (Netea *et al.*, 2019). As a result of this dichotomy, both immune responses are mediated by unique subsets of immune cells. Innate immunity involves the engagement of phagocytes (macrophages, neutrophils), dendritic cells (DCs), mast cells, basophils, eosinophils, and natural killer (NK) cells. In contrast, the adaptive or acquired immune response is mediated by two highly specialized cell types, namely the T and B cells (Warrington *et al.*, 2011). The interplay between those 2 lines of defense and the large variety of involved cells ensure protection against all pathogenic microorganisms encountered during lifetime (de Visser and Coussens, 2005).

Thrombocytes represent the 3rd major group of cells in the hematopoietic system and are known to serve as 'guardians of the vasculature' in case of injured blood vessels, thereby preventing blood loss. Like erythrocytes, platelets do not have a nucleus, however, they possess a complex internal structure characterized by many organelles, granules, and mitochondria. Despite their complexity in the cellular composition, platelets have a diameter of approximately 2 μm and are consequently the smallest cell population circulating in the human body. Due to their small size, thrombocytes are pushed to the periphery by larger blood cells (e.g., RBCs and WBCs), thereby remaining close to blood walls and enabling a rapid reaction in case of vascular damage (Semple, Italiano and Freedman, 2011).

Each of the aforementioned cell types is unique and has specific functions within the hematopoietic network. It is therefore even more fascinating that this variety of cells originates from a single, multi-potent population of stem cells, known as hematopoietic stem cells.

1.2.2. Discovery of hematopoietic stem cells

Every day approximately 500 billion mature blood cells are produced in an adult organism, and each of them is derived from a small pool of multipotent cells termed hematopoietic stem cells (Krause, Scadden and Preffer, 2013). The concept of HSCs began in 1909 when Alexander Maximow envisioned the theory of a lymphocyte-like cell in the bone marrow (BM) that serves as a stem cell for the development of all hematopoietic cells (Maximow, 1909). Maximow's idea encountered much skepticism and remained dormant without attracting scientific interest.

However, in 1961 the work from James E. Till and Ernest McCulloch brought a breakthrough for a new understanding of HSCs. Till and McCulloch provided the first experimental proof for the existence of HSCs with the ability to differentiate into mature cells of the blood lineage. Their functional *in vivo* assay showed that the intravenous injection of bone marrow cells from healthy donor mice into recipients that were previously exposed to irradiation resulted in the formation of multilineage colonies in the spleen, named colony-forming unit-spleen (CFU-S), of transplanted animals (Till and McCulloch, 1961). Follow-up experiments revealed that apart from the multilineage repopulation, colony forming cells also possess a high self-renewal capacity, since CFU-S were shown to originate from a single cell that was able to give rise to similar colonies when injected into a secondary recipient (Becker, McCULLOCH and Till, 1963; Siminovitch, McCulloch and Till, 1963). Initially, CFU-S were considered as the most primitive HSCs due to their characteristic properties, such as self-renewal, multipotency and high proliferative potential. However, subsequent studies identified CFU-S cells in the BM as committed myeloid progenitors and not HSCs (Schofield, 1978; Magli, Iscove and Odartchenko, 1982; Na Nakorn *et al.*, 2002).

The studies of Till, McCulloch and colleagues greatly influenced the understanding of the nature and function of HSCs. Since then, laboratories tried to refine the knowledge and that led to new approaches to study HSCs. In the 1980s, murine studies employing retroviral-based gene transfer were used to investigate the clonal assessment of stem cells. This approach aimed to introduce marker genes into murine hematopoietic progenitor cells through retroviral vectors. It is known that retroviruses integrate at a random site into the genome, thereby generating a specific integration site that serves as a specific marker for each stem cell and its progeny. Consequently, the cells were tagged with a clonal marker that enabled to identify hematopoietic precursors and to track their progeny through differentiation. This method confirmed the existence of a primitive precursor cell that gives rise to myeloid and lymphoid blood cells (Dick *et al.*, 1985; Keller *et al.*, 1985; Lemischka, Raulet and Mulligan, 1986). However, this labeling approach is invasive and may alter the nature of HSC. Nowadays, technological advances, such as the availability of antibodies and fluorescence-activated cell sorting (FACS), as well as the knowledge about molecular surface markers specific for HSCs facilitate a selective purification of the cells for a better understanding of HSC function and the involved molecular regulatory networks (Cheng *et al.*, 2020).

1.2.3. Hematopoietic hierarchy

Hematopoietic stem cells are doubtless the most well-characterized adult stem cells in the human body. Consequently, the extensive research in this field has paved the way for the routine use of HSCs in the clinics, making HSCs the first stem cell type to treat disorders. The fact that HSCs can regenerate all cell types which comprise the blood-forming system led to the formulation of a hierarchical model of hematopoiesis (Fig.2). The classical tree-like branched diagram describes the differentiation pathway of HSCs as a stepwise process that starts from a multipotent HSC, which successively goes through multiple progenitor stages to give rise to terminally differentiated cells (Fig.2a). Since HSCs are capable of reconstituting the entire hematopoietic system, they are located at the apex of this model (Zhang *et al.*, 2018). The small HSC pool is divisible into 2 functionally distinct HSC populations, namely long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs). LT-HSCs are described as highly self-renewing, rare cells that reside in a quiescent state in the BM and possess the ability of long-term hematopoietic reconstitution (~3-4 months) (Morrison and Weissman, 1994; Osawa *et al.*, 1996). Upon receiving stimuli from the surrounding (e.g., in case of inflammation, DNA damage, metabolic stress), LT-HSCs can differentiate to ST-HSCs. Although ST-HSCs have a more restricted self-renewing potential, they possess the unique ability to rapidly repopulate the entire hematopoietic system (Yang *et al.*, 2005). However, in the revised hematopoietic hierarchy model the existence of a new HSC type, termed intermediate-term HSC (IT-HSC), has been proposed (Fig.2b). This transitory cell population, located in-between the LT-HSCs and ST-HSCs, has been shown to be numerically dominant over LT-HSCs and cell transplantation assays in mice revealed that IT-HSCs can contribute to the hematopoietic reconstitution for 6 to 8 months (Benveniste *et al.*, 2010; Yamamoto *et al.*, 2013).

In the classical view, the immediate progeny of ST-HSCs are multipotent progenitors (MPPs) which have a decreased capacity for self-renewal but still possess, as their name suggests, their multidirectional differentiation potential. Although the MPP population was considered as a homogeneous group, the view changed over the years. According to the current state of knowledge, the MPPs are more heterogeneous than initially expected and can be further subdivided into 4 distinct subsets (MPP1-4) based on their immunophenotype, cell cycle activity, lineage bias and bone marrow abundance. Investigators have shown that ST-HSCs and the MPP1 subpopulation possess a similar multiple-lineage reconstitution ability, while the other subsets are linked to different lineage-biased potentials. MPP2 was reported to be a megakaryocyte primed MPP subset, whereas MPP3 functions to rebuild the myeloid compartment. The MPP4 cluster, also known as the lymphoid-primed multipotent progenitor

cells (LMPPs), retains the lymphoid differentiation capacity, thereby giving rise to the common lymphoid progenitor (CLP) (Adolfsson *et al.*, 2005; Cabezas-Wallscheid *et al.*, 2014; Pietras *et al.*, 2015; Wilson *et al.*, 2008). In contrast, as classically suggested, the homogeneous population of MPPs gives rise to oligopotent progenitors, referred to as CLPs and common myeloid progenitors (CMPs) (Kondo, Weissman and Akashi, 1997; Akashi *et al.*, 2000). CMPs are restricted to the myeloid lineages and advance either to the megakaryocyte-erythroid (MEPs), that produce megakaryocytes or erythrocytes, or granulocyte-macrophage progenitors (GMPs), that differentiate to granulocytes and macrophages (Akashi *et al.*, 2000). The lymphoid genesis pathway is essential for the production of T-cells, B-cells, and natural killer (NK) cells, which are direct descendants of CLPs (Kondo, Weissman and Akashi, 1997).

In view of the continuous refinement and the rapidly increasing number of novel concepts and data regarding the HSC differentiation pathway, the prevailing model has been challenged. The idea of HSCs going through defined stages to ultimately differentiate into functionally mature blood cells is increasingly superseded by a model that considers hematopoiesis as a continuous differentiation process devoid of clear intermediate differentiation stages.

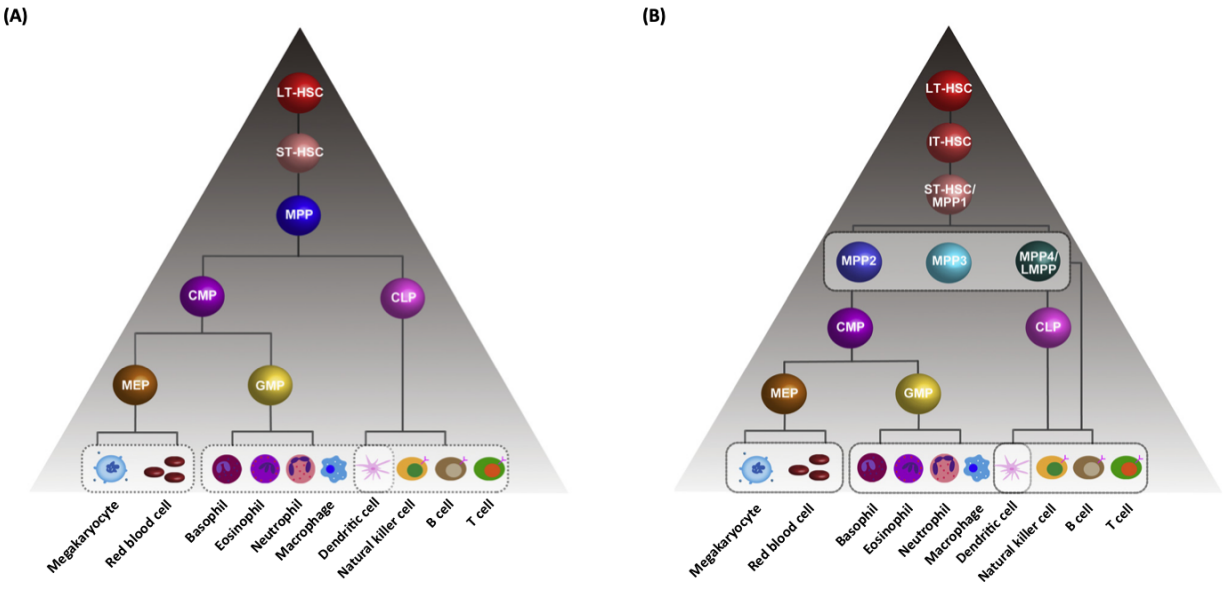


Figure 2. Hematopoietic hierarchy models. **| a.** At the top of the classical model, the bone marrow-residing LT-HSCs differentiate into the ST-HSCs, which further advance to MPPs with restricted self-renewal potential. The first lineage branching occurs when MPPs generate CMPs and CLPs, which differentiate in the next step to lineage-committed effector cell lines. **| b.** In the revised model, HSCs and MPPs are suggested to be more heterogeneous populations. Additionally, the branching into CMPs and CLPs occurs further downstream in the hierarchy (adapted from Y. Zhang *et al.*, 2018).

1.2.4. Hematopoietic stem cell niche

Hematopoietic stem cells, the architects of hematopoiesis, sustain the enormous production of relatively short-lived blood cells during the entire life, simultaneously replicating to give rise to daughter cells with equivalent capabilities to prevent HSC depletion (Ng and Alexander, 2017). To govern a robust and durable blood production, the fate choice of HSCs must be tightly regulated. HSCs are faced with several cell fate options, including self-renewal, differentiation, mobilization, apoptosis, and quiescence, to cope with the hematologic needs of the organism (Lunger, Fawaz and Rieger, 2017). Despite the tremendous knowledge about the biology of HSCs, the exact mechanisms orchestrating fate decisions of these cells remain still elusive. Particular attention is paid to the understanding of regulatory mechanisms involved in the balance between HSC self-renewal and lineage differentiation. Throughout the years, it became clear that this balance depends on a complex interplay of intrinsic and extrinsic factors from the surrounding microenvironment. Many components were identified as critical regulators of stem cell activity, including cytokines, growth factors, transcription factors, epigenetic and cell cycle regulators (Krause, 2002). Furthermore, the bone marrow HSC niche emerged as a key player in the exogenous regulation of HSC fates. In the niche, HSCs receive signals through intercellular communication and various secreted factors from adjacent cells (Morrison and Scadden, 2014; Yu and Scadden, 2016). As the detailed description of all so far known regulators would go beyond the scope of this introduction, the focus will therefore lie on the bone marrow HSC niche and its components that have been shown to affect HSC function.

Based on the studies of Till and McCulloch, who provided evidence for HSCs, Ray Schofield articulated the concept of a stem cell niche in 1978. He recognized differences in the proliferative potential of spleen colony-forming cells and hematopoietic stem cells and suggested that a physiological microenvironment, named niche, preserves HSC potency (Schofield, 1978). According to his view, HSCs reside in the bone marrow, which is the primary anatomical site for HSC production and maintenance, and once HSCs leave this unique compartment, they lose their multipotentiality. This concept planted the seed of scientific curiosity that became a topic of major importance in stem cell research.

The bone marrow is a multifunctional tissue, whose main function is to preserve an HSC reservoir for the continuous production of blood cells. The BM niche can be divided into the endosteal and vascular/perivascular niches, which are not mutually exclusive, but rather exist as overlapping microenvironments (Kiel and Morrison, 2008). Primitive HSCs were initially believed to occur in the endosteal regions of the bone marrow in close contact to osteoblasts

(Nilsson, Johnston and Coverdale, 2001; Xie *et al.*, 2009). Nowadays, it is widely accepted that active ST-HSCs are preferentially enriched in the perivascular region, which contains a network of mesenchymal stromal cells and endothelial cells, while LT-HSCs are located adjacent to the endosteum (Kiel *et al.*, 2005; Kiel *et al.*, 2007). Aside from the maintenance of HSCs, the hematopoietic tissue must also dynamically respond to physiological stimuli in the body, including injuries, infections, and inflammations. The confrontation with cellular stress is often accompanied by the activation of HSCs for subsequent myeloid or lymphoid differentiation, which is of crucial importance to elicit an appropriate stress response (Pietras, 2017; Mitroulis *et al.*, 2020). The variety of supportive cell types found in the BM as well as the factors secreted by each cell type significantly contribute to the regulation of HSCs and the precise role of these distinct cells in hematopoiesis is still a subject of extensive research (Fröbel *et al.*, 2021).

1.2.4.1. Osteoblasts

Bone remodeling, a constant process of resorption and formation of bone tissue, is performed by 2 mesenchymal cell types: the bone-degrading osteoclasts and the bone-forming osteoblasts, a cell type of the endosteal niche (Hadjidakis and Androulakis, 2006). Demonstration of the supporting role of osteoblasts (OBs) in growth and expansion of HSCs in culture, proposed their function as essential contributors to hematopoiesis (Taichman *et al.*, 1996). However, there was a need to evaluate these findings *in vivo*. In 2003, Calvi and colleagues showed that transgenic mice expressing constitutively active parathyroid hormone receptors had an increased number of osteoblastic cells, and the assessment of the bone marrow mononuclear cell fraction revealed elevated counts of HSCs through Jagged 1-mediated Notch activation (Calvi *et al.*, 2003). Another group also provided evidence for the role of OBs in HSC regulation by inhibiting the bone morphogenetic protein (BMP) signaling. The deletion of the BMP receptor type IA (BMPRIA) *in vivo* resulted in an increased number of OBs as well as HSCs (Zhang *et al.*, 2003). Moreover, Visnjic *et al.* demonstrated by using a transgenic mouse model expressing herpes simplex thymidine kinase in OBs that inducible depletion of osteoblastic cells through ganciclovir leads to a reduction in HSCs in the BM at the same time increasing extramedullary hematopoiesis in the liver and spleen (Visnjic *et al.*, 2004). In addition, osteoblastic cells produce a variety of soluble growth factors and cytokines that contribute to HSC fate decisions, such as osteopontin (OPN), angiopoietin-1 (Ang-1) or thrombopoietin (TPO) (Arai *et al.*, 2004; Stier *et al.*, 2005; Yoshihara *et al.*, 2007). On the other hand, depletion of niche factors secreted by OBs, such as the C-X-C motif chemokine ligand 12 (CXCL12) or stem cell factor (SCF) appeared to have barely any effects on HSCs (Greenbaum *et al.*, 2013;

Ding and Morrison, 2013; Ding *et al.*, 2012). Interestingly, osteoblast-specific depletion of CXCL12 but also the direct ablation of OBs *in vivo* affected B lineage progenitors, thereby suggesting an integral role of osteoblasts in B-cell lymphopoiesis (Visnjic *et al.*, 2004; Ding and Morrison, 2013; Greenbaum *et al.*, 2013). Based on the findings presented above, osteoblasts seem to be among the niche cell types that create a supportive microenvironment for HSCs, however, the exact mechanisms by which OBs contribute to HSC regulation still remain largely unidentified.

1.2.4.2. Endothelial cells

Endothelial cells (ECs) comprise a very heterogenous cell population and their phenotypes vary between different tissues. ECs form a monolayer that lines blood vessels and their primary function is the regulation of the vascular homeostasis. The endothelial lining is formed by junctional adhesion molecules and governs a selective transport and exchange of molecules between blood and tissues. Apart from the function as a barrier, the large surface area of the endothelium serves as a platform for cellular interactions (Michiels, 2003; Dejana, 2004). Bone marrow ECs have been shown to express a unique composition of numerous cell adhesion molecules and extracellular matrix (ECM) molecules that influence HSC mobilization and retention (Klamer and Voermans, 2014). The knockout of the adhesion molecule E-selectin, which is exclusively expressed by ECs in the BM, resulted in increased HSC quiescence and self-renewal, demonstrating that E-selectin drives the proliferation of HSCs. Moreover, the deletion or blockade of E-selection was associated with an increased survival of HSCs after treatment with chemotherapeutic agents or irradiation (Winkler *et al.*, 2012). The importance of ECs in the BM niche was further underlined by conditional deletion of the cytokine receptor subunit gp130 in ECs of mice. Despite normal hematopoiesis throughout the fetal and neonatal periods, it could be shown that transplantation of wild-type (WT) BM into irradiated gp130-deficient mice could not rescue the hematologic defects, while transplantation of gp130-deficient BM into irradiated WT mice conferred normal hematopoiesis. This observation demonstrated that gp130-mediated signaling in ECs significantly contributes to hematopoiesis (Yao *et al.*, 2005). ECs have been proposed to express and secrete factors that contribute to hematopoiesis. Ding *et al.* have shown *in vivo* that conditional deletion of the SCF, a key niche component for HSC maintenance, in endothelial results in remarkably reduced HSC frequencies in the BM and diminished reconstitution capacity upon transplantation. Noteworthy, deletion of SCF from both ECs and Leptin-receptor ($LepR^+$) expressing perivascular cells showed an almost complete loss of HSCs, suggesting ECs and $LepR^+$ cells

as major sources of SCF in the BM (Ding *et al.*, 2012). Further, EC-specific production of CXCL12 has been shown to play a role in the maintenance of HSC repopulating activity. Greenbaum and colleagues examined the effect of CXCL12 deletion from different stromal cell populations in the BM using transgenic mice. They were able to demonstrate that loss of CXCL12 in endothelial cells had no effect on the number of HSCs, however, it was associated with a reduction of the long-term repopulating activity (Greenbaum *et al.*, 2013). In line with that, Kobayashi *et al.* showed that Akt-activated endothelial cells upregulate the expression of angiocrine factors (e.g., FGF2, IGFBP2, DLL1, BMP4) thereby promoting the expansion of HSCs and self-renewal of LT-HSCs, whereas MAPK-activated ECs favor differentiation of HSCs into mature hematopoietic cells through downregulation of HSPC-active factors (Kobayashi *et al.*, 2010). Taken together, ECs in the vascular niche provide a wide range of molecular signals that affect HSC function through both direct and indirect mechanisms.

1.2.4.3. Nestin-expressing cells

The understanding of the function of mesenchymal stem cells (or mesenchymal stromal cells; MSCs) in the BM was for a long time hampered by the absence of specific surface markers. The identification of the intermediate filament Nestin (Nes) as a marker for a MSC population named Nestin-expressing MSCs (Nes^+ MSCs) enabled studies of their role in HSC maintenance. Analysis of cells expressing GFP under the regulatory elements of the Nestin promotor *in vivo* showed that Nes^+ MSCs were exclusively present in the perivascular region of the BM. Moreover, they were found to reside in close physical association with HSCs and adrenergic nerve fibers. The contribution of Nes^+ MSCs to the regulation of HSCs depends on the high expression of key molecules for HSC maintenance, such as CXCL12 and SCF, and the depletion of Nes^+ cells results in a significant reduction of bone marrow HSCs, underlining the importance of Nestin-expressing cells in the HSC niche (Méndez-Ferrer *et al.*, 2010).

1.2.4.4. CXCL12-abundant reticular cells

Another cell type of the mesenchymal origin suggested as an important player in hematopoiesis includes CXCL12-abundant reticular (CAR) cells. CAR cells are broadly distributed throughout the BM and mainly present adjacent to sinusoids, which are sites of dynamic cell trafficking between the BM and the blood stream. Studies have demonstrated that CAR cells exert their impact on HSCs through the secretion of high amounts of CXCL12 and SCF. The *in vivo* role of CAR cells was studied using CXCL12-diphteria toxin receptor (DTR) mice that allowed inducible and selective ablation of CAR cells. The absence of CAR cells led to a

reduction in the number and cell size of HSCs and a shift towards a quiescent HSC phenotype. CAR cell-depleted mice had also remarkably reduced levels of lymphoid and erythroid progenitors, indicating the involvement of CAR cells in lymphoid and erythroid lineage development. Further, the authors argue that the mechanism by which CAR cells support HSCs and hematopoietic progenitors depends on the production of CXCL12 and SCF, since protein expression of SCF and CXCL12 was severely reduced in the BM from CXCL12-DTR-GFP mice. This supports the hypothesis that CAR cells are the major producers of these two niche factors, which are crucial for the maintenance for HSCs but also hematopoietic progenitors (Omatsu *et al.*, 2010).

The precise regulation of HSCs within the bone marrow remains a research topic of high complexity. This unique microenvironment comprises different, partially redundant, cellular niche constituents that ensure the functional preservation of HSCs. In addition to cell-cell-interactions, cells convey instructions for HSCs through the secretion of a wide variety of factors (e.g., cytokines, chemokines, angiogenic factors, growth factors). Cellular interactions and various extracellular cues work therefore in concert to orchestrate the function, fate, and behavior of HSCs (Fig.3).

The above description of selected cell types of the BM niche that extrinsically regulate HSCs only scratches the surface of the available information on this cellular network, omitting several other non-hematopoietic cell populations, such as adipocytes or non-myelinating Schwann cells, but also hematopoietic cell types, including megakaryocytes or macrophages. Despite the immense number of studies that aim to unravel the molecular mechanisms and components that control HSCs in their niche, many aspects are still under debate. However, there are no doubts that the crosstalk between HSCs and the various niche cell types is crucial for harnessing the potential of HSCs, and dysregulation of this regulatory network may lead to severe malignancies, including leukemia.

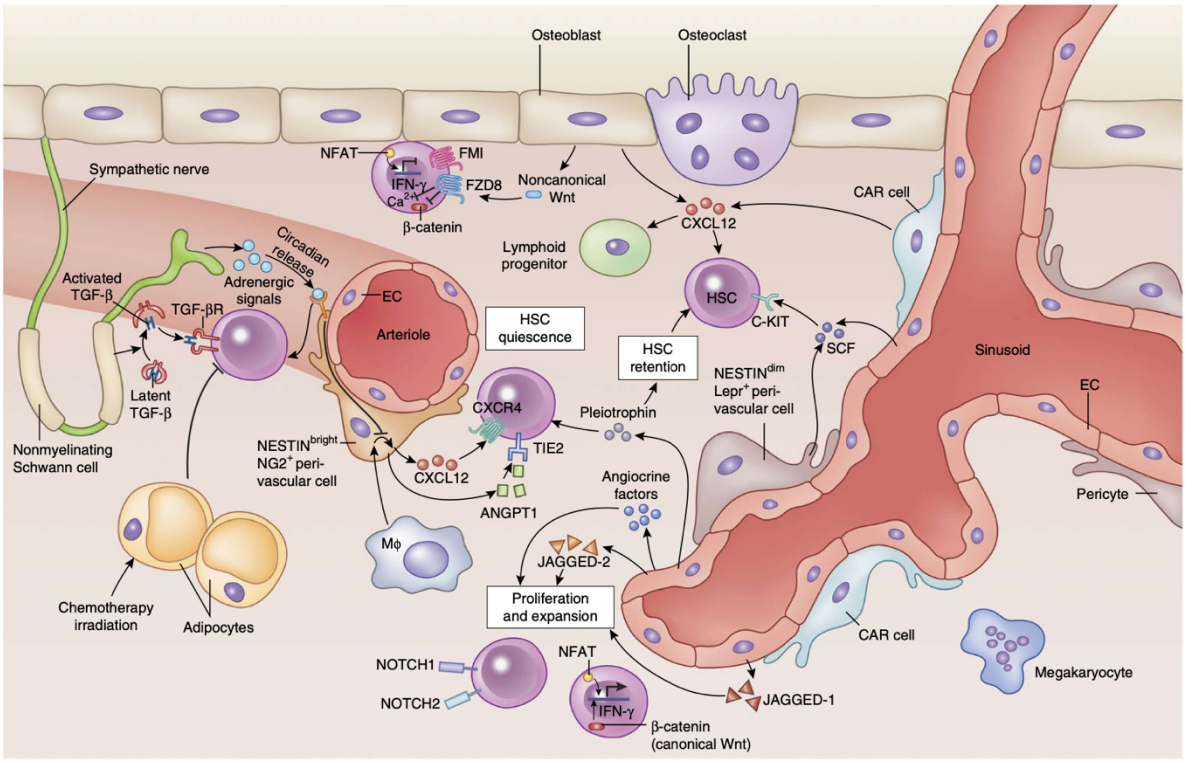


Figure 3. Signaling networks within the bone marrow niche. HSCs receive a plethora of signals from non-hematopoietic and hematopoietic cells that regulate their cell fate and maintenance. Various secreted factors, physical interactions but also signaling pathways act as signals for the regulation of the HSC niche (adapted from Mendelson & Frenette, 2014).

1.3. Clinical application of hematopoietic stem cells

1.3.1. Hematopoietic stem cell transplantation

As one of the best characterized adult stem cells, HSCs are the only stem cell type that is routinely used in the clinics to treat many malignant diseases. During the last 60 years, more than one million patients with hematological malignancies received an HSC transplantation (Chabannon *et al.*, 2018). The purpose of HSC-based therapies is to rescue BM failure after intensive myeloablative chemotherapy or large-field irradiation. The infusion of healthy HSCs rescues the damaged hematopoietic tissue by engrafting in the BM microenvironment and reconstituting the hematopoietic system. HSC transplantation (HSCT) includes grafting with autologous (patient's own cells), syngeneic (cells from an identical twin) or allogeneic (cells from a non-identical donor) stem cells and the decision which variant best meets the patients' needs depends on the donor availability and transplantation indication (Tuthill and Hatzimichael, 2010). First HSCT was conducted using stem cells derived from the BM, but stem cells can also be obtained from peripheral blood or umbilical cord blood (Sorrentino, 2004).

The conclusions drawn from observational studies in mice have paved the way for HSCT in humans. Scientists could demonstrate that the intravenous injection of cellular marrow suspensions after a lethal dose of radiation could rescue the mice (Barnes *et al.*, 1956). The era of human HSCT began in 1957 with the first allogenic bone marrow transplant program by E. Donnall Thomas (Thomas *et al.*, 1957). However, at that time little was known about the role of immunological compatibility between donors and recipients, and as it later turned out this has been the major reason for the prevailing complications accompanied with allogeneic transplants and the poor transplant outcomes. In 1959, Thomas *et al.* reported that in case of syngeneic transplantations between identical twins the BM from the healthy twin was able to restore the hematopoietic system of the leukemic twin, although leukemia recurred in both treated patients (Thomas *et al.*, 1959). This was in accordance with mouse transplantation experiments, which showed increased leukemia relapses following syngeneic transplantations (same mouse strain) compared with allogeneic transplantations (different mouse strain), thereby indicating a role of the donor's immune-mediated response against leukemic cells (Barnes *et al.*, 1956). In 1965, Mathé and colleagues showed that donor T cells, and probably also other immune cells, eliminate recipients' leukemic cells. This phenomenon is referred to as the graft-versus-leukemia (GvL) effect. Moreover, they have also described the "secondary syndrome" (known as graft-versus-host disease [GvHD]), which involved the skin, gastrointestinal tract, and liver (Mathé *et al.*, 1965). These findings showed that the success of

allogeneic transplantations is based on the histocompatibility (or human leukocyte antigen [HLA] matching) between donors and recipients. The selection of compatible donors based on HLA-matching together with immunosuppressive drugs allowed to reduce the risk of immune reactivity of donor cells against the recipients' cells, referred to as graft-versus-host disease (GvHD), but also the host-versus-graft reaction (HvGD) (Thomas, 1999). Therefore, a successful HSCT does not only require donor HLA-matching, but also a pretransplant conditioning, which is a crucial component of the HSCT procedure. The conditioning regimen given prior to an allogeneic HSCT has the purpose of creating sufficient graft space for donor stem cells, preventing graft rejection by suppressing the host immune system and ablating the underlying malignancy (Juric *et al.*, 2016). Improvements in patient selection and introduction of conditioning regimens as a pharmacologic prophylaxis of GVHD led to increased survival rates of transplant recipients and enabled the transition of HSCT from an experimental protocol to an established, widely used and promising treatment in the field of hematology and oncology (Müller, Huppertz and Henschler, 2016).

1.3.2. Sources of stem cell transplants

Nowadays, HSC transplantations are considered as a standard of care for patients with malignant diseases, but also many benign disorders, such as sickle cell anemia, immunodeficiency syndromes or autoimmune diseases (Sorrentino, 2004). Over the past 30 years, from 1990 to 2019, allogeneic HSCTs have increased from 2137 to 19798, while autologous HSCTs have increased from 2097 to 28714 according to the survey of the European Society for Blood and Marrow Transplantation (EBMT) (Passweg *et al.*, 2021). However, the ability to perform allo-HSCTs is often limited by the availability of an HLA-matched donor. In a best-case scenario, the patient has an HLA-identical sibling donor that is considered as the best option for an allogeneic HSCT. Unfortunately, given the decline in family size in western countries, the likelihood of having an HLA-matched sibling donor (MSD) is less than 30 % (Kekre and Antin, 2014). Since the minority of patients have HLA-matched siblings, transplantations using a fully HLA-matched unrelated donor (MUD) are considered a second-tier donor source. The likelihood of finding a MUD in donor registries varies between 16 % and 75 % depending on the ethnicity (Ayuk and Balduzzi, 2019). Despite the immense number of registered volunteer donors, the chance of finding a perfect HLA-match among unrelated donors will decrease with time due the increasing ethnic diversity. Therefore, most of the patients must make use of alternative donor sources, which include HLA-haploidentical, related

donors, partially HLA-mismatched unrelated donors (MMUD), or umbilical cord blood stem cells (Kekre and Antin, 2014).

The first transplantations were performed using bone marrow-derived HSCs, however, today it is known that HSCs can be also found in the peripheral blood (PB) and umbilical cord blood (UCB). The number of HSCs in the BM was shown to be up to 100 times higher compared to the counts detected in the PB under steady-state conditions (Sheridan *et al.*, 1992). However, the administration of granulocyte colony-stimulating factor (G-CSF) to donors prior to stem cell collection induces the mobilization and concentration of HSCs in the PB, leading to HSC numbers that equal or even exceed the concentration in the BM (Metcalf, 1990). Similar outcomes could be observed when patients were treated with chemotherapy without administration of G-CSF (Richman, Weiner and Yankee, 1976). Furthermore, the combination of G-CSF and chemotherapy in autologous donors has been shown to remarkably increase the mobilization effectiveness of $CD34^+$ cells (Akard *et al.*, 1999). Nevertheless, the search for more potent mobilization regimen is driven by two main issues: first, poor mobilization rates in approximately 40 % of the patients and second, the need of high dose of $CD34^+$ cells for rapid engraftment, with ~ 5×10^6/kg being optimal (Pelus and Broxmeyer, 2018).

Peripheral blood as a source of stem cells for clinical use has several advantages over BM transplantations (BMT) including easy and less invasive collection procedure, lower risk of tumor cell contamination and greater quantities of HSCs, leading to faster hematologic recovery. Even though mobilized peripheral blood is currently the most common source of HSCs, especially for autologous transplantations, the use of PB HSCs for allogeneic transplantations is associated with a high risk for GvHD, since PB also contains lymphocyte subpopulations that are responsible for immunological reactivity. On the other hand, recipients benefit from donor T cells that mediate the GvL effect, which is crucial for the elimination of malignant cells. A possibility to avoid a chronic GvHD is the depletion of T cells from stem-cell grafts, resulting in better tolerance of transplants but also in a decreased GvL effect. The choice of an optimal stem cell source for an allogeneic transplantation is therefore more challenging and requires careful weighing of the risks and benefits associated with different stem-cell transplants for each patient (Jansen *et al.*, 2005).

An alternative stem cell source for allogeneic transplantations has been discovered in the 1980s by Broxmeyer and colleagues, who identified the potential of HSCs isolated from human UCB. In 1988, Broxmeyer and Gluckman successfully transplanted UCB-derived HSCs from a healthy, HLA-matched sibling into a patient suffering from Fanconi anemia, a rare, inherited blood disorder. The patient achieved complete hematologic reconstitution and survived without

disease relapse and graft rejection (Gluckman *et al.*, 1989). A few years later, the outcomes of 25 successive transplantations using UCB HSCs from partially HLA-mismatched, unrelated donors showed that despite HLA-disparity, the infused HSCs engrafted and reconstituted the hematopoietic system in almost all transplant recipients. These findings demonstrated that UCB HSCs can provide a useful source of stem cells for clinical utility (Kurtzberg *et al.*, 1996). Now, more than 30 years later, UCB emerged as a common graft source for patients without a matched donor. The reason therefore is that UCB stem cells are immunologically more naïve than HSCs derived from other sources, which means that a lower degree of HLA-matching between the donor and recipient is required for successful transplantation (Ballen, 2017). Consequently, UCB transplants are used for the treatment of a wide variety of disorders and up to date over 40 000 UCB transplants have been performed worldwide. Moreover, the establishment of private and public cord blood banks with about 5 million UCB units enabled a rapid availability of UCB stem cells and significantly reduced the waiting time for transplantations, which is especially important in cases of rapidly progressing disorders. As a result, transplantations can be scheduled according to the patient needs instead of donor availability (Ballen *et al.*, 2015; Dessels *et al.*, 2018; Gupta & Wagner, 2020).

1.3.3. Umbilical cord blood transplantation: Pros & Cons

Hematopoietic stem cells derived from UCB possess many compelling advantages over their counterparts obtained from adult tissues, such as BM or PB. These differences make UCB a useful source of stem cells for cell therapy (Barker, 2007). Among the advantageous qualities of UCB HSCs are their higher proliferation and expansion potentials as well as greater self-renewal capacity compared to HSCs derived from BM or PB, which may result in enhanced homing efficiency and hematopoietic reconstitution capacity of UCB $CD34^+$ cells. Possible explanations for these differences between UCB and adult tissue HSCs are seen in the increased telomere length and higher expression of certain cell cycle regulators observed in UCB-derived HSCs (Mayani, 2010).

As already mentioned before, UCB is an especially promising source of stem cells for patients without matched donors, due to the greater tolerance of donor-recipient HLA-disparity. Most importantly, HLA-matching is a crucial determinant for the outcome of allo-HSCT and the development of GvHD, which is the major cause of morbidity and mortality after allogeneic HSCT. Throughout the years, numerous studies demonstrated that GvHD occurs less frequently after UCBT compared to BMT (Rocha *et al.*, 2000, 2004; Barker *et al.*, 2001). Barker *et al.* investigated the development of GvHD and the survival rate of patients, who received UCBT

from unrelated donors with up to 3 mismatches and BMT from HLA-MUD (6/6 antigen match), and showed that both parameters were comparable between those groups (Barker *et al.*, 2001). A comparison study conducted by Eapen *et al.* demonstrated that 5-year leukemia free survival was similar in patients receiving BMT from HLA-MUD (8/8 match) and UCBT from unrelated donors with 1 or 2 mismatches. Best post-transplantation outcomes were visible in patients after transplants with 6/6 HLA-matched (HLA-A, B, DRB1) UCB with 6 % treatment related mortality (TRM), 34 % relapse, 60 % disease-free survival and 63 % overall survival. For comparison, recipients of fully HLA-matched BMT showed with 19 % treatment related mortality (TRM), 41 % relapse, 38 % disease-free survival and 45 % overall survival (Eapen *et al.*, 2007). This data suggests that mismatched UCB grafts from unrelated donors are a legitimate alternative to HLA-matched BMT in urgent cases.

The exact reason for the less stringent requirement for donor-recipient HLA-matching remains unknown, but it may be attributable to the naïve immunological profile of UCB. Immune cells within cord blood display a naïve, antigen-inexperienced state with limited immunological memory. Studies have demonstrated that T-cells in UCB are characterized by a reduced expression of genes involved in T-cell activation as well as a decreased production of cytokines and chemokines compared to PB-derived T-cells, suggesting a functional immaturity of T-cells in UCB (Kaminski *et al.*, 2003; Chen, Cohen and Lewis, 2006). Since donor T-cells are the primary cells responsible for the pathophysiology of GvHD, the immaturity of UCB T-cells may explain the increased immunological tolerance to alloantigens. In addition, it is known that regulatory T cells (Tregs) occur not only with greater frequency in UCB than in adult PB, but they also possess a higher potential for expansion and increased suppressive functions compared adult Tregs (Taylor, Lees and Blazar, 2002; Mayer *et al.*, 2012). Studies using mouse models have shown that *ex vivo* activated and expanded Tregs were able to induce tolerance after transplantation and prevent GvHD, at the same time preserving the graft-versus-tumor effect (Cohen *et al.*, 2002; Taylor, Lees and Blazar, 2002; Edinger *et al.*, 2003). Thus, UBC-derived Tregs may serve as a potential target for the treatment of acute and chronic GvHD in allogeneic transplantations. Dendritic cells obtained from UCB also display an immature state characterized by decreased antigen-presenting activity, reduced expression of co-stimulatory molecules on the cell surface and lower production of effector cytokines (Drohan *et al.*, 2004). The tolerogenic nature of immune cells in UCB makes them possible targets for the prevention or treatment of GvHD, the major obstacle for therapeutic efficacy of allo-HSCT (Kim and Broxmeyer, 2011).

Despite the beneficial properties of UCB transplantations (UCBT) represented by prompter availability, lower immunogenicity, greater tolerance of HLA-disparity and reduced risk of GvHD, patients may also suffer from several complications that can occur after UCBT (Kindwall-Keller and Ballen, 2020). The use of UCB transplants from mismatched unrelated donors is coupled to several concerns and one of them is the increased risk of infections. Since the immunological memory of immune cells (e.g., B- and T-cells) in UCB is very limited, transplant recipients often suffer from opportunistic infections during the early post-transplant period, due to the lack of pathogen-specific memory T-cells. The lack of transferred adaptive immunity in UCB may be a double-edged sword. On the one hand the naïve profile of immune cells (especially T-cells) decreases the risk of a severe GvHD, but on the other hand the same fact increases the susceptibility of UCB recipients to infectious complications (Montoro *et al.*, 2016).

Another factor that contributes to the increased incidence of post-transplant infections is the delayed immune reconstitution, which is largely due to the small number of infused HSCs. Even though the concentration of HSCs is higher in UCB compared to e.g., PB, the total nucleated cell (TNC) dose in each cord blood unit is lower compared to the other graft sources (Danby and Rocha, 2014). This limits the widespread use of UCB in the clinics, since units with a minimum number of 2.5×10^7 TNC/kg patient body weight are barely available for adult recipients (Scaradavou *et al.*, 2013). This is a major problem since it is well known that the number of transplanted cells per kilogram of body weight positively correlates with transplant outcome (Migliaccio *et al.*, 2000; Gluckman *et al.*, 2004). The small cell numbers that can be obtained from UCB are thus only sufficient to treat children. Due the lower TNC dose in UCB compared to BM or PB, the time to neutrophil and platelet engraftment is prolonged. While the median time of neutrophil recovery after UCBT is 30 days, neutrophil engraftment after BMT and PB HSCT is achieved on day 21 and 14, respectively (Seggewiss and Einsele, 2010). The recovery of platelets after BMT, PB HSCT and UCBT can be observed after 24 (to 28), 18 and 44 days, respectively (Kimura *et al.*, 2013). Since the low stem cell dose is the major factor responsible for the delayed engraftment, several approaches were developed to overcome this hurdle. In order to hasten the engraftment, the idea of transplantations with double UCB units arose. The major benefit of this strategy is the infusion of an increased cell dose, which might improve the engraftment in transplant recipients. Verneris *et al.* compared in a study the outcomes of single and double cord blood transplants (dUCBTs) in adults suffering from acute leukemia with a special focus on the risk of disease relapse. The findings showed that the incidence of sustained engraftment was similar between single- and double-unit recipients with

no significant differences in neutrophil and platelet recovery (Verneris *et al.*, 2009). These findings were in accordance with those previously reported by Brunstein and colleagues (Brunstein *et al.*, 2007). A noteworthy aspect of the study conducted by Verneris *et al.* is that the use of 2 partially HLA-matched UCB units for transplantation was associated with significantly lower leukemia relapse compared to patients that received only 1 partially HLA-matched UCB unit. This effect was suggested to be linked with an enhanced GvL effect due to the greater degree of HLA-disparity. Interestingly, the incidence of GvHD and the overall survival was comparable between patients receiving single and double UCB transplants, implicating that infusion of 2 UCB units in patients with acute leukemia might be an option for future treatments (Verneris *et al.*, 2009). Other strategies to overcome the hurdle of the limited cell numbers in UCB include reduced intensity conditioning, combined use of cord blood and haploidentical transplants, intra-bone UCB graft injection, co-infusion of supportive cell types (e.g., MSCs) and *ex vivo* expansion of UCB HSCs (Stanevsky, Goldstein and Nagler, 2009; Danby and Rocha, 2014).

1.3.4. *Ex vivo* expansion of cord blood-derived stem cells

The limitations associated with the use of UCB grafts represented by slow engraftment and delayed immune reconstitution due to the limited HSC numbers led to the idea of *ex vivo* expansion of UCB-derived HSCs. The infusion of *ex vivo* expanded cells has the goal to improve the engraftment and hasten the hematologic recovery, thereby counteracting the early morbidity and mortality after UCBTs. To date, many different experimental approaches aimed to increase the number of UCB HSCs being transplanted. However, despite encouraging results, many laboratories worldwide are still in search for optimal conditions to achieve a satisfying level of expansion without undesired side effects (Flores-Guzmán, Fernández-Sánchez and Mayani, 2013).

1.3.4.1. Cytokine-mediated HSC expansion

Since HSCs reside in the hematopoietic niche surrounded by many different cells that have a crucial impact on HSC location and function (e.g., by secretion of regulatory cytokines), successful *ex vivo* HSC cultures require conditions that recapitulate this *in vivo* environment. One common denominator for HSC cultures is the presence of cytokines that are imperative for hematopoietic cell growth. Thus, initial expansion attempts included the supplementation of culture medium with various combinations of hematopoietic cytokines. Piacibello *et al*. demonstrated that the use of an *in vitro* system containing a combination of Fms-like tyrosine

kinase 3 ligand (FLT3L) and TPO was able to sustain the proliferation and self-renewal of UCB-derived $CD34^+$ cells over a period of 6 months (Piacibello *et al.*, 1997). Others have shown similar results, thus enabling to draw the conclusion that the presence of the 3 early-acting cytokines FLT3L, TPO and SCF is essential for the proliferation of primitive hematopoietic cells *in vitro* (Gilmore *et al.*, 2000; Tanavde *et al.*, 2002). Despite the initial enthusiasm, transplantation of *ex vivo* expanded $CD34^+$ cells cultured in the presence of FLT3L, TPO and SCF resulted in a modest progenitor cell expansion (4-fold increase) and did not improve the engraftment kinetics (Shpall *et al.*, 2002). Similar results were reported by other groups that also investigated the *in vivo* engraftment of cells expanded in the presence of various other cytokine mixtures (Jaroscak *et al.*, 2003; De Lima *et al.*, 2008). Apart from cytokines, several other soluble factors have been also shown to affect HSC proliferation, such as CC-chemokine ligand 28 (CCL28), Insulin-like growth factor 2 (IGF-2) or pleiotrophin (Karlsson *et al.*, 2013; Zhang & Lodish, 2004; Himburg *et al.*, 2010).

1.3.4.2. Notch-mediated HSC expansion

Neighbouring cells within the niche do not only influence HSCs by the secretion of cytokines but also by the expression of different ligands on their cell surfaces, which initiate signalling cascades in human HSCs. One pathway that has been shown to be a key regulator of HSC self-renewal is the NOTCH signalling pathway (Stier *et al.*, 2002). Researchers tried to exploit the ectopic activation of the NOTCH signalling as a target for *ex vivo* expansion of HSCs. For this purpose, a Notch ligand consisting of the extracellular domain of DELTA1 coupled to the Fc-region of the human immunoglobulin 1 (IgG1) was engineered (Varnum-Finney *et al.*, 2000). The incubation of UCB-derived $CD34^+$ cells with the engineered Delta-like ligand and cytokines (SCF, TPO, Flt3L, IL-3, IL-6) has resulted in significant increase in the number of HSCs. Furthermore, the transplantation of Notch-expanded $CD34^+$ cells into the BM of immunodeficient nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice resulted in a 16-fold enhanced repopulating potential and a rapid reconstitution of the myeloid compartment (Delaney *et al.*, 2010). In a phase I clinical trial, patients receiving infusions of Notch-expanded $CD34^+$ cells together with unmanipulated grafts showed an enhanced rate of myeloid engraftment and a significantly shortened time to neutrophil recovery compared to the control group. Even though preliminary results from this trial indicated that the Notch-mediated *ex vivo* expansion system for human HSCs might be potentially effective way to enhance engraftment kinetics, the neutrophil recovery was shown to be transient and the majority of the

expanded cells possessed just a short-term repopulating ability (Delaney *et al.*, 2010; Papa, Djedaini and Hoffman, 2020).

1.3.4.3. Co-culture of HSCs with stromal cells

Another experimental expansion strategy that aimed to mimic the HSC niche complexity was based on co-cultures of HSCs with stromal cells. As already described in chapter 1.2.4., the HSC niche is composed of multiple non-hematopoietic cell types, which provide molecular cues that influence HSCs cell fate decisions (Fuchs, Tumbar and Guasch, 2004). By culturing HSCs *ex vivo*, the cells are devoid of supporting cells as well as their extrinsic input and rely only on the conditions provided by culture medium. To, at least partially, counteract this lack of cell-to-cell contact, researchers started to culture HSCs in the presence of various stromal cell types. Mesenchymal stromal cells belong to the cell subset that contributes in a meaningful way to the orchestration of the HSC niche. MSCs exert their effects on HSCs by expressing several ligands (e.g., Jagged-1, N-cadherin) that activate essential signalling pathways in HSCs, but also by secreting a whole bunch of paracrine factors (e.g., SCF, CXCL12), which play a pivotal role for HSC regulation and maintenance (Crippa *et al.*, 2021). Given the HSC supportive role of MSCs within the BM niche, MSCs have been employed in the clinical setting for *ex vivo* expansion of HSCs. Indeed, co-culturing of unfractionated UCB mononuclear cells with mesenchymal cells has been shown to enhance the expansion of primitive and mature hematopoietic progenitors, thus providing evidence for the beneficial effect of MSCs on HSC survival and proliferation (Robinson *et al.*, 2006). Furthermore, infusion of UCB HSCs co-cultured with MSCs in combination with unmanipulated UCB into patients demonstrated improved engraftment associated with faster neutrophil and platelet recovery compared to patients, who received unmanipulated UCB only (De Lima *et al.*, 2012). The use of the MSC-based co-culture system has been shown to enhance HSC expansion in a safe and effective way. However, the restricted capacity of *ex vivo* expanded MSCs to home and engraft in the BM niche is one of the functional characteristics that limits the widespread use of this expansion strategy (Crippa *et al.*, 2021).

1.3.4.4. Small molecule-mediated HSC expansion

The list of small molecules for *ex vivo* expansion of HSC has been considerably extended over the last few years. However, one may wonder why it's necessary to search for additional compounds that drive HSC expansion when cytokines or other growth factors have already shown to be capable of expanding HSCs; the problematic aspect with the use of various

combinations of nutritional complements and cytokines is that despite their addition to culture medium shows initial HSC expansion, the self-renewal and reconstitution capacity of HSCs cannot be sustained, and the hematopoietic differentiation pathway is initiated. This led to the search for small-molecule chemical compounds, which interact with different targets, thus promoting HSC proliferation. To date, numerous molecules have been described as applicable compounds for *ex vivo* HSC expansion (e.g., NR-101, zVADfmk, zLLYfmk, Nicotinamide, Resveratrol, TEPA, dmPGE2, Garcinol, serotonin) (Ghafouri-Fard *et al.*, 2021). Notably, in 2014 the research group led by Guy Sauvageau identified UM171, a pyrimido-indole derivative, as an HSC expansion-promoting compound (Fares *et al.*, 2014). Since then, UM171 has gained increasing attention as a novel agonist for HSC renewal. Few years later, Subramaniam *et al.* were able to uncover the mechanism of action of the small molecule. UM171-mediated HSC expansion was found to be associated with the lysine-specific histone demethylase 1A (LSD1) and RCOR1, a subunit of the CoREST complex. It was shown that UM171 exert its effects by promoting proteasomal degradation of LSD1 and RCOR1, which both restrict the expansion of HSCs (Subramaniam *et al.*, 2020). In agreement with this, Chagraoui et al. complemented these results by demonstrating the involvement of the CUL3/KBTBD4 ubiquitin ligase in the degradation of LSD1 and RCOR1 upon UM171 treatment (Chagraoui *et al.*, 2021). The acquired knowledge of how UM171 mechanistically triggers the *ex vivo* expansion of human UCB HSCs brings the researchers one step closer to its future application in the clinics.

1.3.4.5. Identification of HSC regulators using RNAi

Aside from extrinsic modulation, such as supplementation with various cytokines or co-culturing of HSCs with stromal cells, another approach for expansion of functional HSCs *ex vivo* includes the silencing of regulators, which restrict HSC self-renewal and differentiation. One efficient and convenient way to identify potential negative regulators of hematopoiesis is to target a candidate gene using viral vector-mediated gene silencing by RNA interference (RNAi) (Fig.4a). This reverse genetic approach enables to study the effects of gene silencing by evaluating the associated phenotype. This is an especially feasible technique when working with cells that are characterized by a limited persistence under *ex vivo* culture conditions. More specifically, HSCs are known as cells that preserve their undifferentiated state just for a short time in culture. This makes them a well suitable cell type to study the effects of a specific gene knockdown, since enhanced maintenance or expansion of primitive HSCs in culture would suggests that the targeted gene may play a role in regulating HSC fate decisions. Furthermore,

the evaluation whether the silencing of a candidate gene indeed has a beneficial effect can be easily determined by the expression of cell surface markers, which can be traced by FACS (Ali *et al.*, 2009).

The way to the development of an efficient and applicable system to study the effects of gene silencing on HSC function has been hampered for a long time by low levels of gene transfer into the cells. Initially, oncoretroviral (RV) vectors were mainly used for gene transfer into HSCs, however, even though the gene-transfer efficiency was high in case of murine HSCs, RV vectors achieved poor transducibilty of human HSCs. Most probably, the reason therefore may be the fact that oncoretroviruses require dividing cells for a successful gene transfer and HSCs are usually maintained in a quiescent, non-proliferating state. Since transduction efficiency remains a crucial factor for successful inhibition (or induction) of the expression of a given gene in biomedical research, the interests focused on lentiviral gene delivery systems. Lentiviruses, such as the human immunodeficiency virus 1 (HIV-1), have considerably improved the transduction efficiency and turned out to be an especially promising gene delivery tool in case of non-diving cells, such as HSCs. Nowadays, lentiviral delivery of RNAi constructs is a widely used and well-established method for the assessment of gene function in primary mammalian cells (Richter and Karlsson, 2001; Woods, Ooka and Karlsson, 2002). Furthermore, the application of lentiviral-based delivery systems for the treatment of several genetic diseases has been shown to be a safe method with a low risk of random integration into the hosts genome and no adverse effects related to the lentiviral vector (Cartier *et al.*, 2009; Cavazzana-Calvo *et al.*, 2010; Sessa *et al.*, 2016). Based on the results from these studies, it can be concluded that the use of a lentiviral-mediated gene therapy of HSCs has a great potential for providing clinical benefits.

The steadily increasing knowledge about RNAi and its growing potential for medical applications has led to the emergence of RNAi-mediated gene silencing as a powerful, experimental method to dissect functional aspects of HSCs. This approach can be expanded to perform genome-wide RNAi screens to identify genes that are molecular regulators of self-renewal and differentiation in hematopoiesis. Using large-scale RNAi screens, several regulators of both murine and human HSCs could be identified (Hope *et al.*, 2010; Baudet *et al.*, 2012; Cellot *et al.*, 2013; Kinkel *et al.*, 2015; Galeev *et al.*, 2016; Sertorio *et al.*, 2017). RNAi may therefore be a valuable tool for enhancing the intrinsic self-renewing properties of HSCs *ex vivo*, which could be used in a clinically compliant manner to generate a safe and efficacious way to overcome material shortage for stem cell transplantations (Fig.4b).

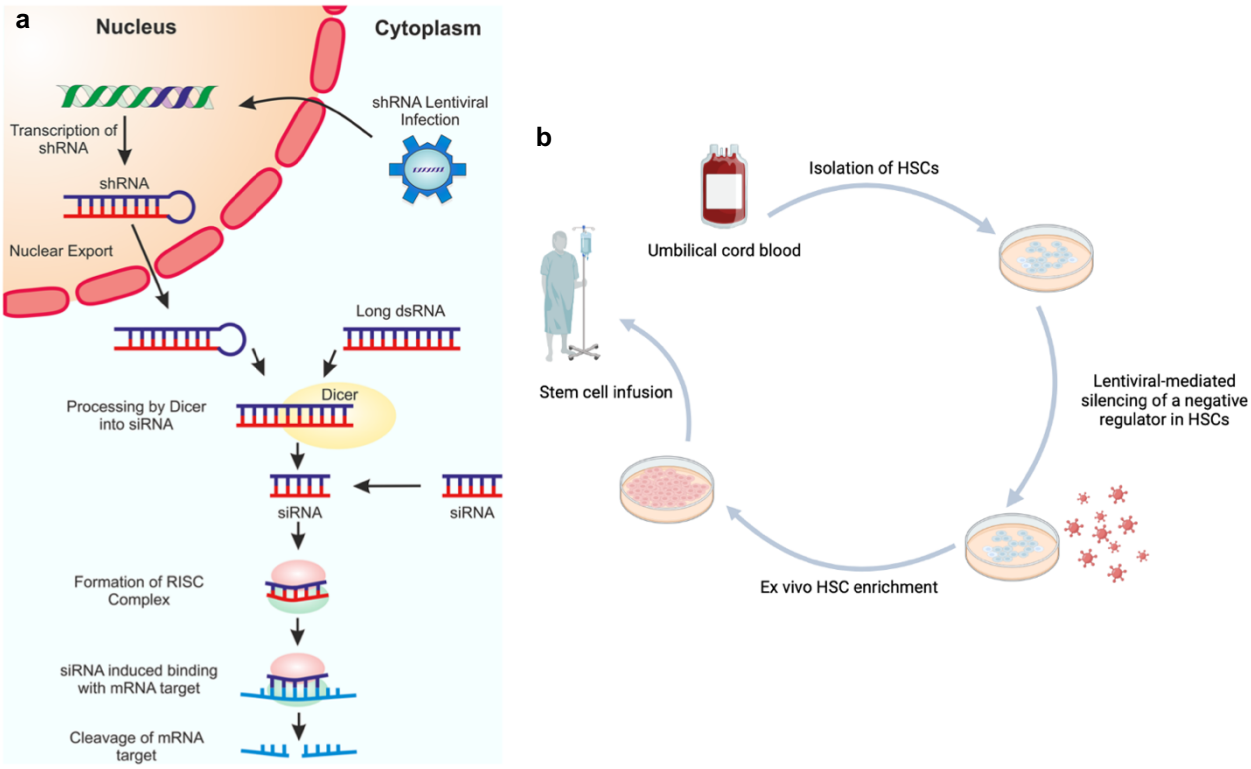


Figure 4. RNAi-based approach in HSCs | **a**. Schematic representation of the RNAi pathway. A short hairpin RNA (shRNA) is introduced into the cell via viral transduction. Following the transcription in the nucleus, the shRNA is exported to the cytoplasm, where it is processed by Dicer into siRNA. In the next step, the siRNA is loaded into the RISC complex, followed by the removal of the passenger strand. The remaining guide strand directs the RISC complex to the complementary mRNA. Depending on the sequence complementarity, the target mRNA can be either cleaved or translationally repressed. (adapted from Barrass & Butcher, 2020) | **b.** Potential use of RNAi for *ex vivo* expansion of HSCs. The restricted number of HSCs in a single UCB unit could be increased by silencing an intrinsic regulator, which would enable *ex vivo* expansion of HSCs for their subsequent use in the clinics. Created with BioRender.com

1.3.4.6. Gene candidates selected from shRNA screen for individual validation

Based on the results of a primary cell RNAi screen conducted in our laboratory, we decided to select in total 10 gene candidates, whose knockdown was associated with $CD34^+$ proliferative effects, for individual validation. Candidates selected for individual knockdown are involved in diverse biological processes, ranging from transcription regulation to proteins involved in cell migration or RNA processing. Some of them are acting as scaffolding proteins or structural components of larger complexes, thereby providing a platform for the assembly of signalling molecules. To give an overview about the molecular function of selected candidates, a simplified scheme is shown in Fig.5.

Candidates included in the category "signal transduction" are a part of multi-step signalling pathways that regulate crucial processes within the cell. For instance, the **A-kinase anchoring protein 9 (AKAP9)** is a scaffolding protein that binds the regulatory subunit of protein kinase

A (PKA) and directs the kinase to specific locations within the cell, thereby regulating the subcellular compartmentalization of PKA and the phosphorylation state of target proteins (McConnachie, Langeberg and Scott, 2006). In contrast, the precise molecular function of **Caspase 8-associated protein 2 (CASP8AP2)** remains largely unknown. Reports identified CASP8AP2 as a component of the Fas-induced apoptotic pathway, however, it is also indicated to both enhance and suppress the transcriptional activity of steroid hormone receptors (Imai *et al.*, 1999; Kino and Chrousos, 2003; Kino, Ichijo and Chrousos, 2004). The **G-protein coupled receptor 126 (GPR126)** belongs to the group of adhesion GPRs, a yet least studied class of the GPR family, and has been suggested to play an essential role in Schwann cell myelination (Mogha *et al.*, 2013).

The category "RNA processing/transport/binding" comprises candidates which have an impact on protein biosynthesis through the regulation of different RNA molecules. For example, the **nucleolar protein 9 (NOL9)**, a polynucleotide 5′-kinase, is involved in the processing of ribosomal RNA (rRNA) that leads to a mature 60S ribosomal subunit (Heindl and Martinez, 2010). **TSEN15** is a subunit in the human TSEN complex, which catalyzes the removal of introns from precursor tRNAs. The whole complex is composed of 4 subunits, including 2 endonucleases with catalytic activity, TSEN2 and TSEN34, and 2 structural components, TSEN15 and TSEN54. Little is known about the precise role of TSEN15 in splicing, and mutations have been so far only found in patients with pontocerebellar hypoplasia (PCH). Also, the molecular mechanism of pathogenesis causing PCH remains elusive (Budde *et al.*, 2008; Breuss *et al.*, 2016). A candidate involved in the post-transcriptional gene regulation is **UPF3A**, **a regulator of nonsense transcripts 3A**. Nonsense mediated RNA decay (NMD) is a post-splicing mechanism that inhibits translation of messenger RNAs (mRNAs) harbouring a premature termination codon and prevents the generation of truncated, dysfunctional proteins. Recently, Yi et al. showed that UPF3A activates the NMD pathway in cells deficient in its paralog UPF3B (Yi *et al.*, 2022). However, this is contrary to a previous study in which the authors claimed that UPF3A acts primarily as a NMD repressor by sequestering UPF2 from the NMD complex (Shum *et al.*, 2016). The role of UPF3A in NMD is therefore not fully clear and it seems to vary among different species.

The involvement of the **lysine methyltransferase 5A (SETD8 or KMT5A)** in transcription regulation is provided by its ability to specifically monomethylate the lysine residue 20 of histone 4 (H4K20me1), thereby recruiting signalling molecules to DNA double-strand breaks or directly modulating chromatin compaction (Milite *et al.*, 2016). In contrast, little is known about the function of the **zinc finger protein 98 (ZNF98)**. It is most likely that ZNF98 exerts

its effect like the vast majority of ZNF family members by interacting with DNA, RNA or proteins to regulate gene expression and participate in a variety of physiological processes (Cassandri *et al.*, 2017). Finally, **Podocan (PODN)**, a member of the small leucine-rich repeat (SLR) protein family, is indicated to regulate migration and proliferation of smooth muscle cells, thus playing a role in pathogenesis of cardiovascular disease and atherosclerosis (Shimizu-Hirota *et al.*, 2004).

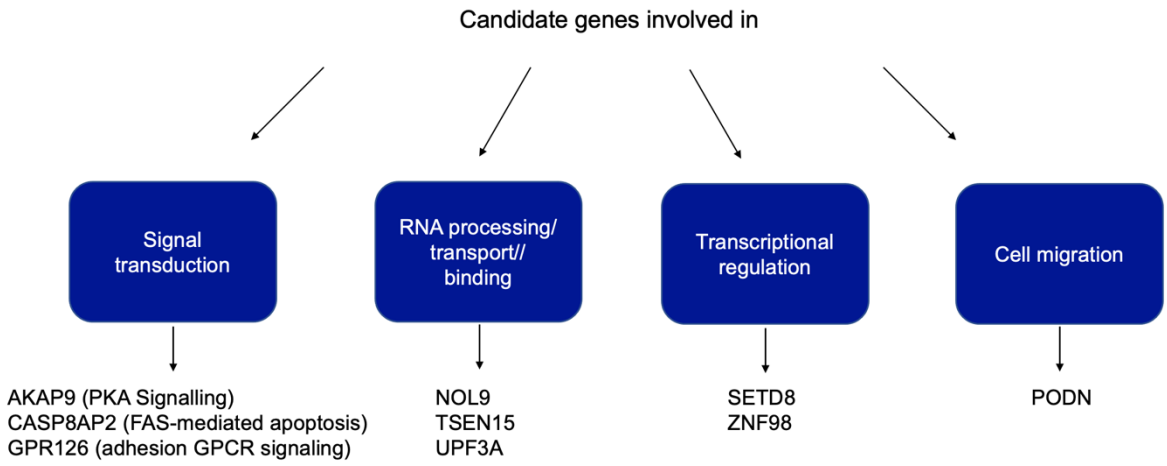


Figure 5. Overview of candidates selected for individual evaluation using lentiviral shRNA-mediated knockdown. Candidates were grouped based on similar molecular functions into 4 categories: Signal transduction, RNA processing/transport/binding, transcriptional regulation, and cell migration.

2. Aim of the thesis

Given their remarkable regenerative potential, HSCs provide a curative treatment option for many patients suffering from hematological malignancies. Once there is a need for a stem cell transplantation, the question about the stem cell donor arises. Especially, in cases in which the patients do not have an available HLA-matched donor in their family, umbilical cord blood transplants are a good alternative graft source of HSCs. However, the major obstacle for the broad use of UCB in the clinics is the limited number of HSCs within a single cord blood unit. Thus, a potential way to overcome this hurdle could be the *ex vivo* amplification of HSCs derived from UCB, which could be transplanted into the patients.

This thesis aims to identify and characterize novel intrinsic regulators of HSC fate decisions, which would promote HSC *ex vivo* expansion. To achieve this objective, 10 candidate genes were silenced using a lentiviral-mediated RNAi approach. The effects of gene silencing were assessed in long-term cultures of HSCs under standard stem cell conditions. Candidate genes, which exhibited an enhanced proliferative potential combined with the maintenance of a primitive phenotype were subsequently subjected to RT-qPCR to evaluate the knockdown efficiency. Based on these results, the list of gene candidates was narrowed down to one candidate gene, which complied with the requirement to be viewed as a hit. Further analysis included assays that aimed to understand functional relevance of the gene in HSCs.

3. Materials and Methods

3.1. Cloning of shRNAs into lentiviral vectors

shRNA sequences (Tab.1) were cloned into pLKO.1-GFP self-inactivating lentiviral vectors, which drive the shRNA expression from a human U6 promoter and carry GFP under control of a phosphoglycerate kinase (PGK) promoter. For the assembly of the insert with the vector, homologous overlap regions were added to the ends of the shRNA sequences by PCR (DreamTaq DNA Polymerase, Thermo Scientific) (Tab.2 and 3). The generation of pLKO.1-EGFP vectors expressing shRNAs was carried out using the Gibson assembly method (NEBuilder HiFi DNA Assembly, New England Biolabs) according to manufacturer's instructions. The construct was transformed into competent *E. coli* cells followed by plating of the transformation mixture on agar plates supplemented with ampicillin (100 µg/ml). On the next day, 2 colonies per shRNA were picked for Miniprep cultures in 5 ml Circlegrow medium (Nordic Biolabs) supplemented with ampicillin (100 µg/ml). Plasmids were extracted using a Plasmid Miniprep Kit (Thermo Scientific) according to manufacturer's instructions and quantified using a NanoDrop spectrophotometer (Thermo Fisher). The extracted plasmids were sequenced to verify the identity of the shRNA. Lentiviruses were produced by the Cell & Gene Therapy Core at the Lund Stem Cell Center. Noteworthy, one of the candidates will be treated anonymously using name candidate X. The information about shRNA target sequences, TaqMan probes or its biological function will not be stated.

Table 1. List of all shRNAs used in this thesis

Name	Target Sequence (sense strand)
Scramble shRNA	CAACAAGATGAAGAGCACCAA
AKAP9 shRNA1	GCTGACTATTTCAGAAGAAAT
AKAP9 shRNA2	CGCAGTGAAGAACGGGATAAA
AKAP9 shRNA3	TATGAACACAGCTTATGATTG
CASP8AP2 shRNA1	GCGAGTCTATACCAGCTTGTA
CASP8AP2 shRNA2	CCAGTCTCTTAAGAAGAATAT
CASP8AP2 shRNA3	GGAAACCAAGTCAACGTTATT
GPR126 shRNA1	GCTCATTCAGACAACTTCTAT
GPR126 shRNA2	CCTATCTTACATCCAAATCTA
GPR126 shRNA3	ACTTAACCTCAGCCAATATTA
NOL9 shRNA1	GCCATTACAATGTACCGAGA

NOL9 shRNA3	ATCGAAGGGACAGTACCTTAT
ZNF98 shRNA1	TATACTTGATGAGAACAAATG
ZNF98 shRNA2	CCCTTACTACACATAAGATAA
ZNF98 shRNA3	TCACACTTAGCTCAACATAAA
UPF3A shRNA1	GCAGAAACCATTCTAAAGAAA
UPF3A shRNA2	GACGTAGAAACACGCAGAAAC
UPF3A shRNA3	GACAGCAAAGGCCTAGAATAT
PODN shRNA1	CAACTTAGAGTTTGGTGACAT
PODN shRNA2	CCAACCTCAATTACCTGTACT
PODN shRNA3	CCTGTACTTGGCCAATAACAA
SETD8 shRNA1	CAAAGGACAAAGTGCCCTCAA
SETD8 shRNA2	GAATCGCAAACTTACGGATTT
SETD8 shRNA3	CGCAACAGAATCGCAAACTTA
TSEN15 shRNA1	GAAGTAAACTGTGTAGGATTA
TSEN15 shRNA2	CCAAGTTTATGTAGCGTTCTT
TSEN15 shRNA3	ACAGTTATATACAGGTTTATG

Table 2. PCR primers for addition of homologous ends

pLKO.1–GFP	**Sequence**
Forward primer (5' – 3')	CTTTATATATCTTGTGGAAAGGACGAAACT
Reverse primer (3' – 5')	ATACTGCCATTTGTCTCGAGGTCGAGAATT

Table 3. PCR program

Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	98°C	4 min	1
Denaturation	98°C	30 sec	34
Annealing	62°C	1 min	
Elongation	72°C	30 sec	
Final elongation	72°C	5 min	1

3.2. Isolation of cord blood derived $CD34^+$ cells

Umbilical cord blood samples were obtained from maternal wards at Skåne University Hospital in Lund and Malmö, as well as from Helsingborg General Hospital in Sweden. UCB was collected into tissue culture flasks with RPMI containing 1% FCS, 1% penicillin-streptomycin

(P/S) and Heparin (5000 IU/ml). For the isolation of mononuclear cells (MNCs), UCB was diluted 1:1 with phosphate buffered saline (PBS) and layered onto Lymphoprep tubes (Alere Technologies). Density-gradient centrifugation was conducted according to manufacturer's instructions. MNCs were subsequently pooled in Dulbecco's modified Eagle's medium (DMEM) and centrifuged at 450g for 5 min. Cells were washed once with PBS supplemented with 2% fetal bovine serum (FBS) and 2 mM ethylenediaminetetraacetic acid (EDTA) (Isolation buffer). $CD34^+$ cells were purified from MNCs using CD34 immunomagnetic beads (Miltenyi Biotec) and MACS cell separation columns (LS Columns, Miltenyi Biotec), following the manufacturer's recommendations. For freezing, $CD34^+$ cells were gently resuspended in 500 µl DMEM with 50% FBS (Freezing medium A) and 500 µl freezing medium A containing 20% dimethylsulfoxide (DMSO, Sigma-Aldrich). Cells were stored at –150°C until use. Noteworthy, a cell batch was usually composed of $CD34^+$ cells pooled from several cord blood units.

3.3. *In vitro* culture & lentiviral transduction

For *in vitro* HSC cultures, cells were thawed and subsequently sorted for $CD34^+$ HSCs by FACS to obtain a homogenous $CD34^+$ cell population. Sorted $CD34^+$ cells were cultured in serum-free expansion medium (SFEM, STEMCELL Technologies) supplemented with 1% P/S and standard growth factors consisting of human SCF, TPO, and FLT3L (each at 100 ng/ml, PeproTech) at 37°C in an atmosphere of 5% CO_2. For lentiviral transduction, UCB-derived $CD34^+$ cells were allowed to recover a few hours from thawing and sorting. Cells (50 000 cells/well/200 µl) were lentivirally transduced overnight in 96-well plates with a target transduction efficiency of 30% to 50%. On the next day, half of the culture medium was removed and replaced with fresh SFEM. Transduced UCB-derived $CD34^+$ cells were analysed by FACS for GFP, CD34 and CD90 expression every week. Cells were split as needed, but at least once a week.

3.4. Colony forming-unit (CFU) assay

To test the colony-forming potential of HSPCs, 300 GFP^+CD34^+ cells were sorted 72h post transduction and plated at 1 ml per well (6-well plates) in Methocult H4230 (STEMCELL Technologies) supplemented with human SCF (25 ng/ml), interleukin-3 (IL-3, 25 ng/ml), granulocyte-macrophage colony-stimulating factor (GM-CSF, 50 ng/ml), and erythropoietin (EPO, 4U/ml). Plates were incubated at 37°C for 12-14 days. The total number of erythroid

(burst-forming unit-erythroid (BFU-E), colony-forming unit-erythroid (CFU-E)) and non-erythroid colonies (CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), CFU-granulocyte, macrophage (CFU-GM), CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM)) was counted manually under a light microscope (Olympus).

3.5. Differentiation assay

To assess the differentiation capability of UCB-derived $CD34^+$ cells, cells were transduced with lentiviruses (day 0) and transferred at day 3 to differentiation medium composed of Myelocult H5100 (STEMCELL Technologies) supplemented with SCF (50 ng/ml), GM-CSF (20 ng/ml), FLT3-L (10 ng/ml), TPO (50ng/ml), IL-3 (20 ng/ml) (Peprotech), 5U/ml EPO (Apoteket Sweden) and Penicillin (100U/ml) Streptomycin (100 µg/ml) (Cytiva). FACS analysis was performed at day 4, 7 and 10 and 14 after the transfer to differentiation medium.

3.6. Flow cytometry & and cell sorting

For phenotypic analysis, cells were collected into a 96-well plate, washed once with isolation buffer, and stained with for 30 min at 4°C. Following antibodies were used for fluorescence-activated cell sorting (FACS) analysis: CD34-PE-Cy7, CD34-BV510, CD90-APC, CD90-BV395, EPCR/CD201-APC, CD11b-APC, CD15-PE-Cy7, CD71-APC. All antibodies were diluted in isolation buffer according to the manufacturer's recommendation. Afterwards, cells were washed once and resuspended in isolation buffer containing 7-aminoactinomycin D (7-AAD, Sigma-Aldrich) prior to analysis. For cell sorting, cells were thawed and subsequently stained with PE/Cyanine7 anti-human CD34 following the same procedure as described above. A BD LSRFortessa and a BD LSRFortessa X-20 (BD Biosciences) were used to analyse samples. Cell sorting was performed using a BD FACSAria IIu (BD Biosciences). Data analysis was performed using the software FlowJo (FlowJo).

3.7. RNA extraction and cDNA synthesis

To assess the knockdown efficiencies on mRNA level, GFP^+ cells were sorted 72h post transduction into RLT lysis buffer (QIAGEN) containing 1% β-mercaptoethanol (Sigma-Aldrich). Total RNA was extracted from $CD34^+$ cells using the RNeasy Micro Kit (QIAGEN) according to manufacturer's instructions. cDNA was synthesised from RNA using Superscript III (Invitrogen) reverse transcriptase protocol. RNA and cDNA were stored at –80°C until required.

3.8. Quantitative real-time PCR

Quantitative PCR (qPCR) for selected candidate genes was performed using TaqMan probes from Applied Biosystems (Tab.4). All qRT-PCR reactions were performed in a total volume of 20 µl containing 1 µl of TaqMan probe, 2 µl of template cDNA, 7 µl of nuclease free water and 10 µl of TaqMan Universal PCR Master Mix (Applied Biosystems). PCR was carried out using the QuantStudio 1 Real-Time PCR System (Applied Biosystems) with the following protocol: initial denaturation at 95°C for 10 min, and 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Samples were normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT) levels and fold change determined by the ΔΔCT method. Noteworthy, qPCR analysis was not performed for AKAP9, NOL9 and ZNF98 candidate genes.

Table 4. List of TaqMan probes used for qRT-PCR

Target gene	Assay ID
HPRT	Hs02800695_m1
CASP8AP2	Hs01594281_m1
GPR126/ADGRG6	Hs01089210_m1
PODOCAN	Hs00956648_m1
SETD8/KMT5A	Hs00360662_s1
TSEN15	Hs00379071_m1
UPF3A	Hs01589834_m1

3.9. Statistical analysis

Statistical significance was calculated in GraphPad Prism 9 (GraphPad Software) using a 2-tailed unpaired student's t-test to analyze groups of 2 or one-way analysis of variance (ANOVA) for the analysis of multiple groups. Unless otherwise indicated, error bars represent standard error of the mean (SEM). P values lower than 0.05 were considered significant.

4. Results

4.1. Individual validation reveals TSEN15 as a potential HSPCs regulator

Based on the results of a previous genome-wide RNAi screen, 10 candidate genes, which positively correlated with HSPC *ex vivo* expansion, were selected to be individually tested using lentiviral shRNA-mediated knockdown (3 shRNA constructs per gene). The lentiviral based system enables long-term gene silencing through the ability to integrate the stem loop shRNA cassette into the host genome. For this purpose, shRNAs were cloned into the viral expression vector pLKO.1-GFP, followed by the production of lentiviral particles that utilize the expression vector. The effects of gene silencing were evaluated in HSPC cultures under standard stem cell conditions. Given the limited persistence of the primitive HSPC phenotype ($CD34^+/CD90^+$) in *ex vivo* cultures, selection of shRNAs, which confer an increased expansion, could be conducted by immunophenotyping. The lentiviral shRNA vector additionally expresses a recombinant GFP protein, which allows to monitor and isolate shRNA-expressing HSPCs after transduction (Fig.6).

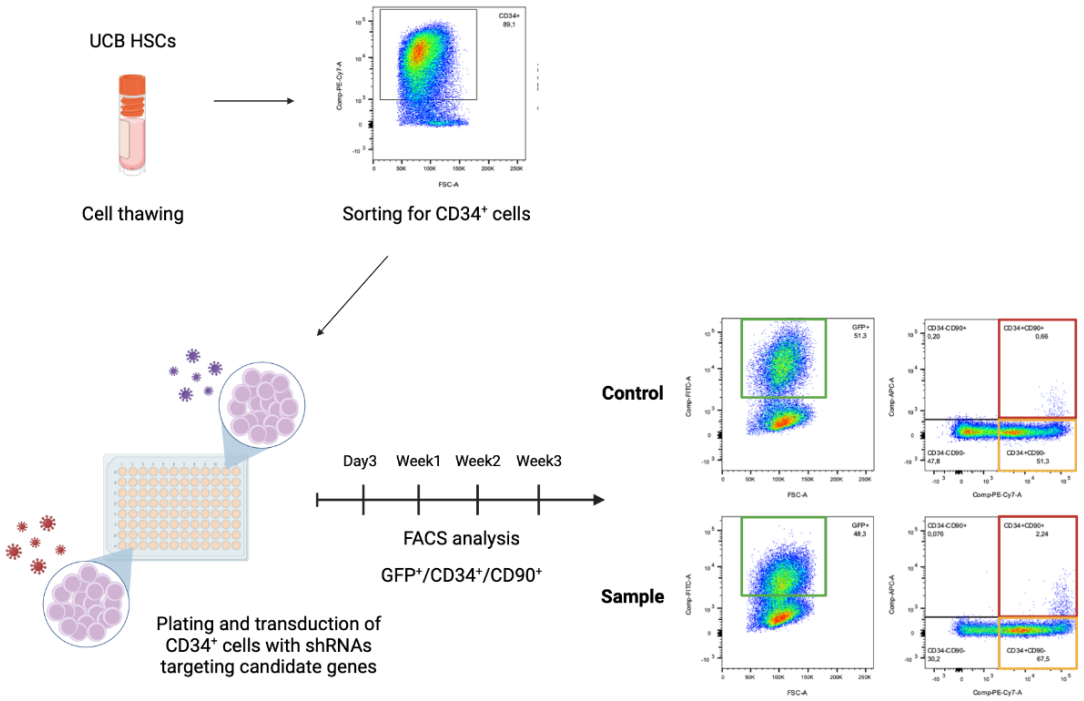


Figure 6. Overview of the experimental setup used to study the effects of gene silencing in long-term HSPC cultures. After thawing, cells were sorted based on CD34 expression and plated in 96-well plates (50 000 cells per well) for transduction with lentiviruses carrying the shRNA expression vectors. The assessment of shRNA effects on HSPC expansion was conducted by weekly FACS analysis for GFP, CD34 and CD90 expression. Created with BioRender.com

The HSPC expansion potential was measured after knocking down the selected candidate genes. Transduced and phenotypically defined HSPCs (GFP^+CD34^+) was significantly increased after 2 weeks of culture in case of candidate X and TSEN15 knockdown (Fig.7a). Candidate X silencing with shRNA2 and shRNA3 resulted in 62% and 71% GFP^+CD34^+ cells respectively, while in case of the scrambled control around 40% of the cells were GFP^+ and $CD34^+$. Similarly, knockdown of TSEN15 with shRNA1 and shRNA2 showed 75% and 64% of GFP^+CD34^+ cells after 2 weeks of culture. In contrast, knockdown of AKAP9, CASP8AP2, GPR126 and ZNF98 led to a reduction of GFP^+CD34^+ cells, indicating that those candidates may be essential for HSPCs. An overview about the expansion of $CD34^+$ and $CD34^+CD90^+$ cell numbers over the period of 3 weeks as well as the knockdown efficiencies of the shRNAs targeting the candidate genes is provided in Fig.S1 and S2. Apart from candidate X and TSEN15, the knockdown of PODN with shRNA1 also showed significantly elevated percentages of GFP^+CD34^+ cells (61%), however this phenotype is most likely represents an off-target effect of the shRNA since displayed only by one shRNA.

Even though CD34 is a well-established selection and enrichment marker for human HSPCs, it is also expressed by a broad range of other cells, such as lineage-committed progenitors but also many non-hematopoietic cell types, including stromal, epithelial or endothelial cells (Sidney *et al.*, 2014). Based on the knowledge acquired from studies on non-human primate stem cell transplantations and gene therapy, it is known that multilineage long-term engraftment and BM reconstitution are driven by the $CD34^+CD90^+$ population, which contains the most primitive HSPCs (Radtke *et al.*, 2017). The inclusion of the CD90 marker allows therefore to validate the impact of the knockdown on actual stem cell renewal more specifically. Tracking the effects of gene silencing in the HSPC-enriched $CD34^+CD90^+$ cell population mostly confirmed the results observed in case of GFP^+CD34^+ cells (Fig.7b). Knockdown of candidate X and TSEN15 significantly increased the percentage of $GFP^+CD34^+CD90^+$ cells after two weeks of culture. Candidate X silencing with all 3 shRNAs led to ~3% $GFP^+CD34^+CD90^+$ cells, while knockdown of TSEN15 increased the percentage to up to ~4%. In addition to those two candidates, also single shRNAs in case of PODN (shRNA3, ~7%) and UPF3A (shRNA1, ~3%) exhibited an increase in the percentage of $GFP^+CD34^+CD90^+$ cells compared to the scrambled control (~1%). Surprisingly, even though shRNA3 targeting PODN shows ~7% of $GFP^+CD34^+CD90^+$ cells, this does not correspond with the results observed in Fig.7a. An increase in $CD90^+$ cells is usually accompanied by an equivalent increase in $CD34^+$ cells, since $CD90^+$ cells also express CD34. Since one of the major concerns associated with the use of

shRNAs are the off-target effects, the selection of candidates for further validation was based on a phenotypic effect that was confirmed by at least two different shRNA sequences.

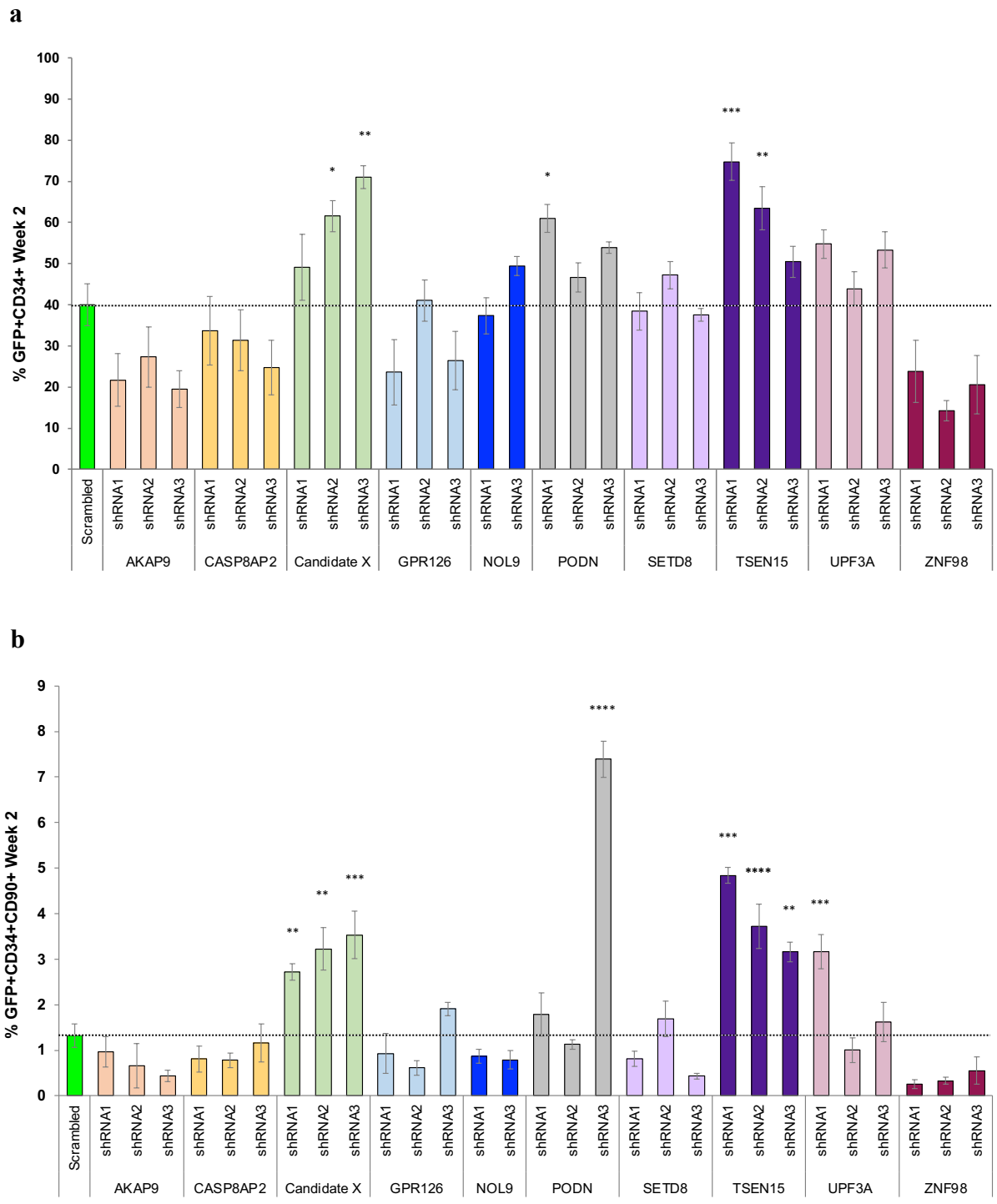


Figure 7. Individual validation reveals TSEN15 as a promising candidate. | **a.** Graph showing all genes and shRNAs used for individual validation and the percentage of transduced cells expressing the surface marker CD34 (GFP^+CD34^+) after two weeks of culture. | **b.** Graph showing all genes and shRNAs used for individual validation and the percentage of transduced, phenotypically defined HSPCs ($GFP^+CD34^+CD90^+$) after two weeks of culture. Results are representative of at least 2 independent experiments with a minimum of 3 technical replicates per experiment. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)

4.2. Knockdown of TSEN15 expands primitive cells *in vitro*

Given the effects of TSEN15 knockdown observed in HSPC cultures, we decided to focus more closely on this candidate and its expansion phenotype. In the first line, knockdown efficiency of each shRNA targeting the TSEN15 gene was assessed. For this purpose, UCB-derived $CD34^+$ cells were transduced and GFP^+ cells were sorted three days after transduction. The data showed that all three shRNAs led to a significant reduction of TSEN15 mRNA levels compared to the control (Fig.8a). The TSEN15 constructs resulted in silencing ranging from approximately 31% to 41%. The scrambled control served as a baseline for changes in the expression levels.

The functional validation of shRNAs targeting TSEN15 in HSPC cultures demonstrated that its knockdown resulted in an increased expansion of both total $CD34^+$ cells and the HSPC-enriched $CD34^+CD90^+$ population compared to the control (Fig.8c-f). $CD34^+$ cells targeted with shRNA1 or shRNA2 expanded ~1.7- and ~1.5-fold, respectively, within 2 weeks compared to the control (Fig.8c). As shown in representative FACS plots, while ~52% of the scrambled shRNA-transduced cells expressed CD34, shRNA1- or shRNA2-transduced cells exhibited a significant increase in CD34 expression after 2 weeks of culture, with ~70% of the cells being CD34 positive (Fig.8e). After 4 weeks, shRNA1 and shRNA2 induced a ~1.6 and 2.2-fold expansion of $CD34^+$ cells, respectively (Fig.8c and 8f). Even though the gene expression analysis showed that shRNA3 reduced the mRNA expression levels of TSEN15, shRNA3 was associated with a decreasing trend in both total $CD34^+$ and $CD34^+CD90^+$ cells, suggesting that shRNA3 may target another mRNA sequence, whose downregulation has toxic effects on HSPCs (Fig.8c-f).

Regarding the $CD34^+CD90^+$ population, TSEN15 knockdown resulted in an expansion of more than ~3- and ~4.5-fold in shRNA1- and shRNA2-transduced cells after 4 weeks of culture, suggesting that decreased expression of TSEN15 on mRNA level promotes the proliferation of $CD34^+CD90^+$ cells (Fig.8d).

Monitoring the transduction efficiency in control and TSEN15-targeted cells enables to draw conclusion about a potential growth advantaged conferred by the different shRNAs. During the period of 4 weeks, the percentage of GFP^+ cells in the control group remained steady at ~50% (± 5%), which was close to the initial transduction efficiency measured at day 3. In contrast, TSEN15-targeted cells, especially shRNA2-transduced cells, which had an initial transduction efficiency of ~45%, exhibited a significant growth advantage with ~67% of cells expressing GFP after 4 weeks of culture (Fig.8b). Cells transduced with shRNA1 demonstrated a slight increase from ~58% (day 3) to ~64% of GFP-expressing cells during a 4-week period.

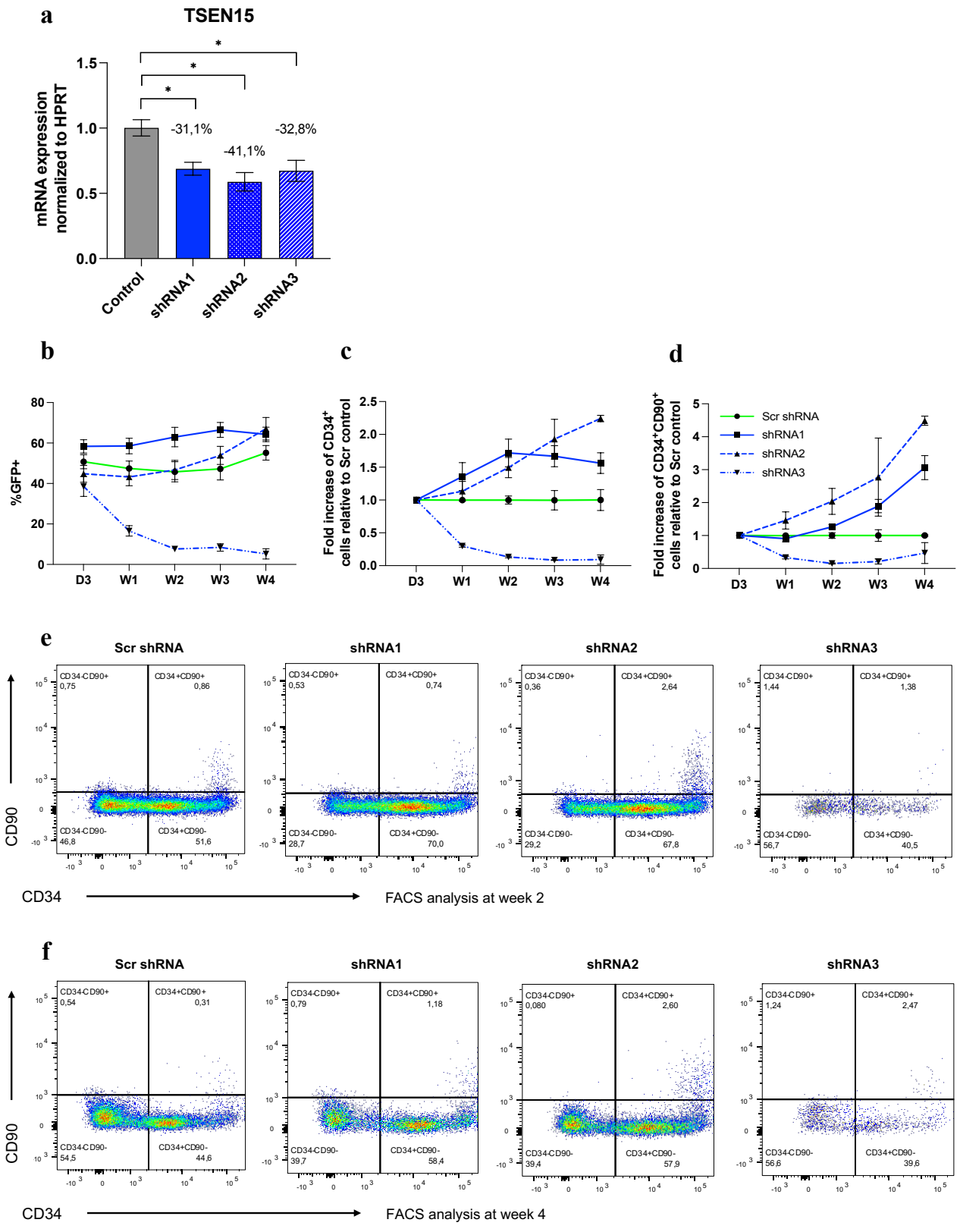
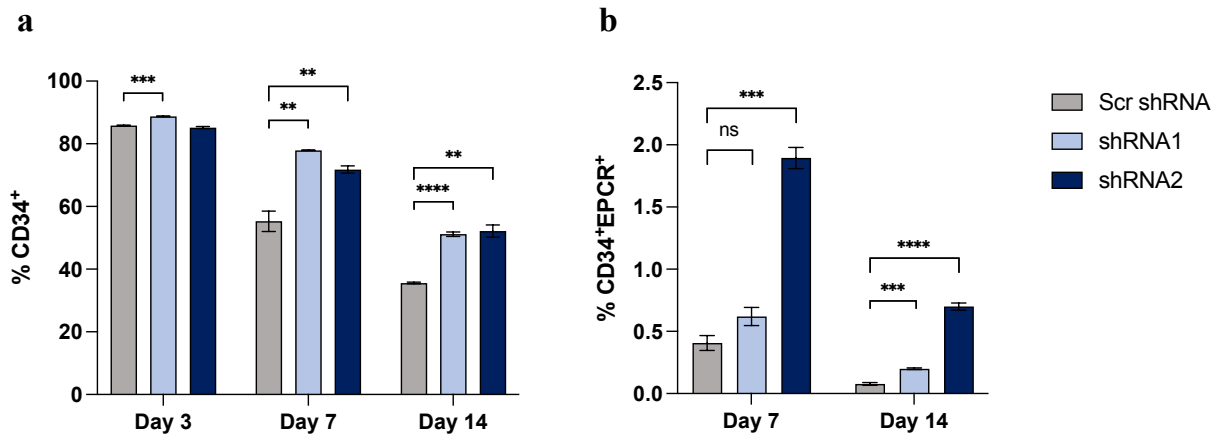


Figure 8. Knockdown of TSEN15 expands primitive cells *in vitro*. | **a.** Knockdown efficiency of shRNAs targeting TSEN15 as measured by qPCR. Data from one experiment with 9 replicates. | **b.** Percentage of GFP^+ cells and expansion of GFP^+CD34^+, $GFP^+CD34^+CD90^+$ cell number over a period of 4 weeks for the TSEN15 gene compared to scrambled control. Data from 2 independent experiments with 3 replicates each. | **c.** Representative FACS plots showing CD34 and CD90 expression after 2 weeks of culture. | **d.** FACS plots showing CD34 and CD90 expression after 4 weeks of culture. (* $p<0.05$). | D, Day; W, Week, Scr, Scrambled.

4.3. TSEN15 knockdown leads to an increase in the $CD34^+EPCR^+$ population

Despite the existence of a reliable combination of surface markers ($CD34^+CD38^-CD45RA^-CD90^+$) for the enrichment of HSPCs, its use for the evaluation of human HSPC activity *ex vivo* is frequently not suitable since the surface phenotype of cultured HSPCs most likely differs from that of freshly isolated cells, as already shown in case of murine HSPCs (Zhang & Lodish, 2005). A few years ago, the endothelial protein C receptor (EPCR), also known as CD201, was suggested as a novel marker of cultured HSPCs (Fares *et al.*, 2017; Subramaniam *et al.*, 2019). Furthermore, EPCR expression has been demonstrated to be highly upregulated in UCB-derived HSPCs treated with UM171, which promotes *ex vivo* expansion of HSPCs (Fares *et al.*, 2017). Based on these findings, we decided to evaluate the expression of EPCR upon knockdown of TSEN15. Noteworthy, as shRNA3 targeting TSEN15 showed a toxic effct on the cultured cells, we decided to conduct further experiments only with shRNA1 and shRNA2. As shown in Fig.9b, knockdown of TSEN15 with shRNA2 resulted in a significant increase in the $CD34^+EPCR^+$ population after 7 days of culture, with ~2% of cells expressing both markers. Even though shRNA1-targeted cells did not exhibit a similar increase at that time point, after 14 days of culture both shRNAs (shRNA1: 0.2%; shRNA2: 0.7%) demonstrated a significantly increased expression of CD34 and EPCR compared to the control (Scr shRNA: 0.08%) (Fig.9b and 9c). Furthermore, knockdown of TSEN15 with shRNA1 and shRNA2 increased the percentage of $CD34^+$ cells over 14 days of culture compared to the control. These results confirm the results presented in the section 4.2.



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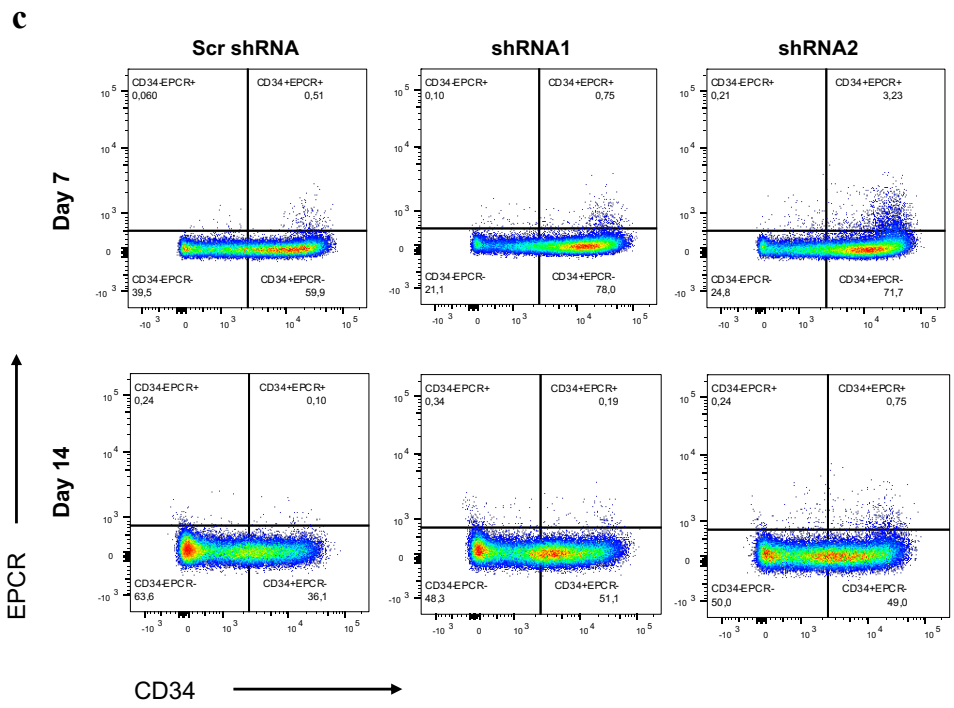


Figure 9. TSEN15 knockdown leads to an increase in the $CD34^+EPCR^+$ population. | **a.** Percentage of $CD34^+$ cells at day 3, 7 and 14. Data are means ± SEM of triplicate wells. | **b.** Percentage of $CD34^+EPCR^+$ cells at day 7 and 14. Data are means ± SEM of triplicate wells. | **c.** Representative FACS plots showing CD34 and EPCR expression on day 7 and 14. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$) | ns, not significant.

4.4. TSEN15 knockdown does not affect the colony-forming ability of HSPCs

After demonstrating that silencing of TSEN15 has an impact on the proliferation of $CD34^+$ and $CD34^+CD90^+$ cells, we next wanted to investigate whether TSEN15 knockdown affects the clonogenic progenitor activity. The functional analysis of hematopoietic stem and progenitor cells (HSPCs) is of particular importance for the selection of cells with the highest quality for subsequent transplantations. The colony-forming unit (CFU) assay serves as a well-established *in vitro* approach to measure the potency of HSPCs to proliferate and differentiate into colonies. Even though the transplantation of HSPCs into an *in vivo* mouse model serves as an ultimate proof-of-concept to assess the functionality of HSPCs, the CFU assay has been shown to correlate well with engraftment success. For this reason, we asked whether TSEN15 knockdown affects the differentiation potential of HSPCs. Therefore, sorted UCB-derived GFP^+CD34^+ cells were seeded 3 days after lentiviral transduction in a semi-solid medium supplemented with a combination of various cytokines, which stimulate the formation of erythroid and myeloid colonies. The results showed that knockdown of TSEN15 with shRNA1 or shRNA2 did not perturb the differentiation potential of HSPCs derived from UCB (Fig.10a and 10b). Although the number of colonies was not significantly different, shRNA2-transduced

cells exhibited a slight increase in the total number of colonies and a greater proportion of myeloid colonies. Notably, the lentiviral transduction of cells itself did not affect the colony forming capacity since non-transduced mock cells had a similar overall score compared to the scrambled control.

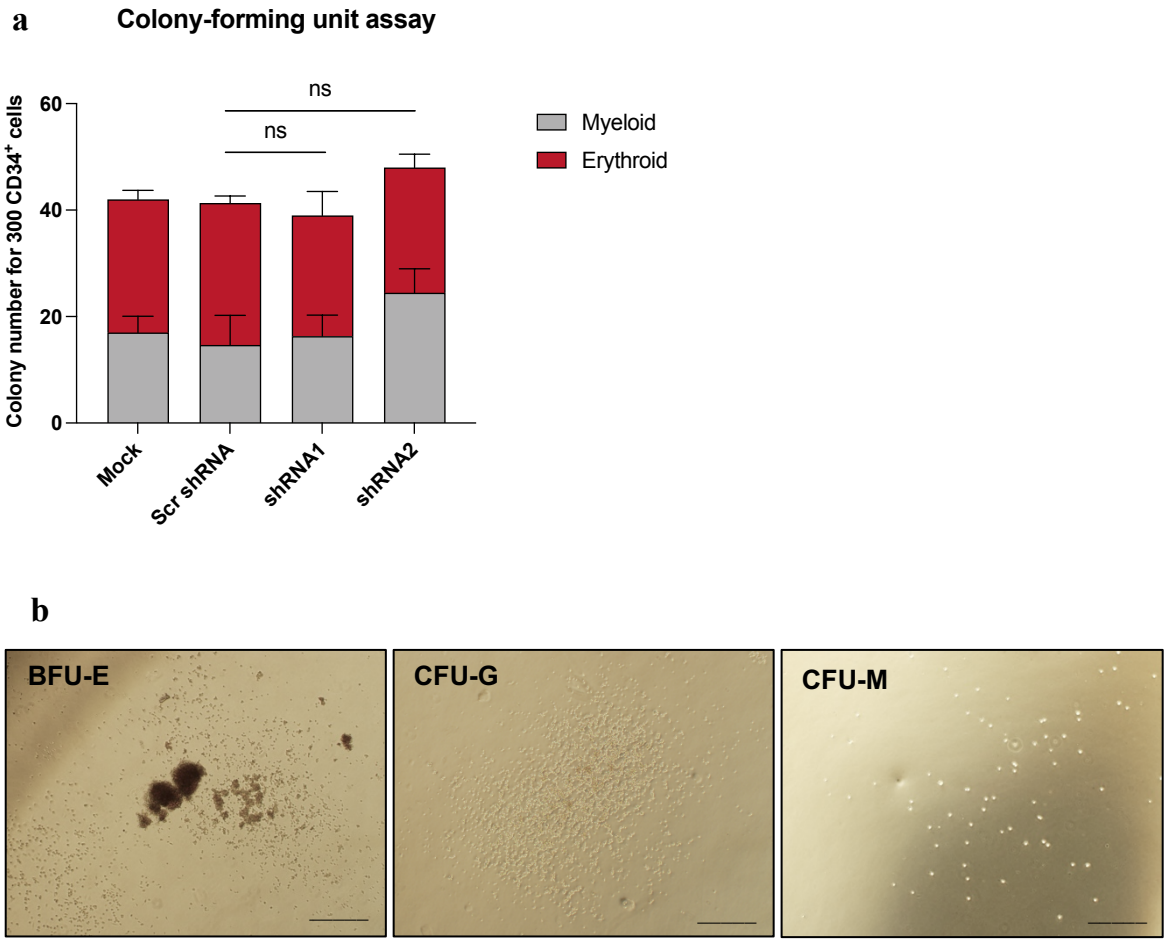
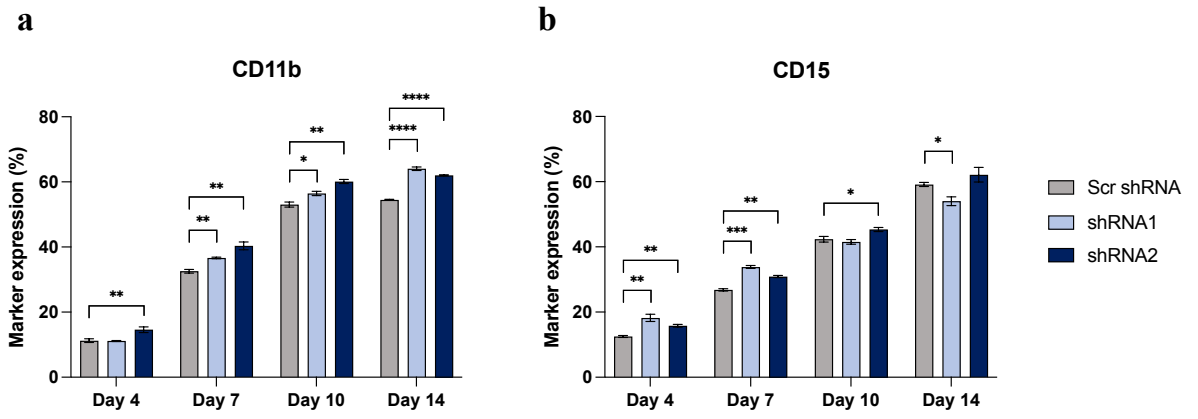


Figure 10. Knockdown of TSEN15 does not affect the colony-forming ability of HSPCs. | **a.** Colony-forming ability of UCB-derived $CD34^+$ cells transduced with Scr shRNA, TSEN15 shRNA1, TSEN15 shRNA2 and non-transduced mock cells. Colony counts were scored on day 14. Data are means ± SEM of triplicate wells. | **b.** Representative microscopy CFU images. Scale bar 100 µm. | ns, not significant.

4.5. Knockdown of TSEN15 upregulates myeloid differentiation of HSPCs

Even though the CFU assay is one of the most extensively used assays to assess the functionality of HSPCs, the results provide just a snapshot of the colony frequencies at a single timepoint. Furthermore, identification of the different colony types in the culture is often subjective and the results can vary from person to person. In order to get a more objective view on the differentiation potential of the hematopoietic progenitors, we performed a differentiation assay in liquid cultures, which allowed us to monitor the differentiating cells at different timepoints. To promote myeloid differentiation, transduced $CD34^+$ cells were grown in liquid media for 14 days. For tracing the cells with a myeloid profile, we used the surface markers CD11b and CD15, which are both expressed during myeloid maturation. CD15 expression is induced in the mid-stages of monocytic and myelocytic differentiation and increases with progressive maturation (Pui, 1995). In contrast, the surface antigen CD11b marks different mature myeloid cells, including monocytes, macrophages, granulocytes, and natural killer cells (Pahl, Rosmarin and Tenen, 1992).

Surprisingly, HSPCs transduced with either TSEN15 shRNA1 or shRNA2 showed already on day 4 an increased expression of both CD11b and CD15 markers compared to the control (Fig.11a-c). As shown in Fig.11a, expression of CD11b was significantly increased up to day 14 in shRNA1- and shRNA2-transduced cells, reaching ~64% and ~62% respectively, compared with ~55% in control cells. In contrast, the expression of CD15 was significantly different in TSEN15-targeted cells compared to the control up to day 7 (Fig.11b and 11c). At day 7, expression of CD15 in cells transduced with shRNA1 or shRNA2 was significantly increased to ~34% and ~31% compared to the control with ~27%. Following the culture period of 2 weeks, the CD15 expression levels started to equalize with the control, indicating that TSEN15 knockdown induces a shift of the myeloid differentiation towards a monocytic lineage.



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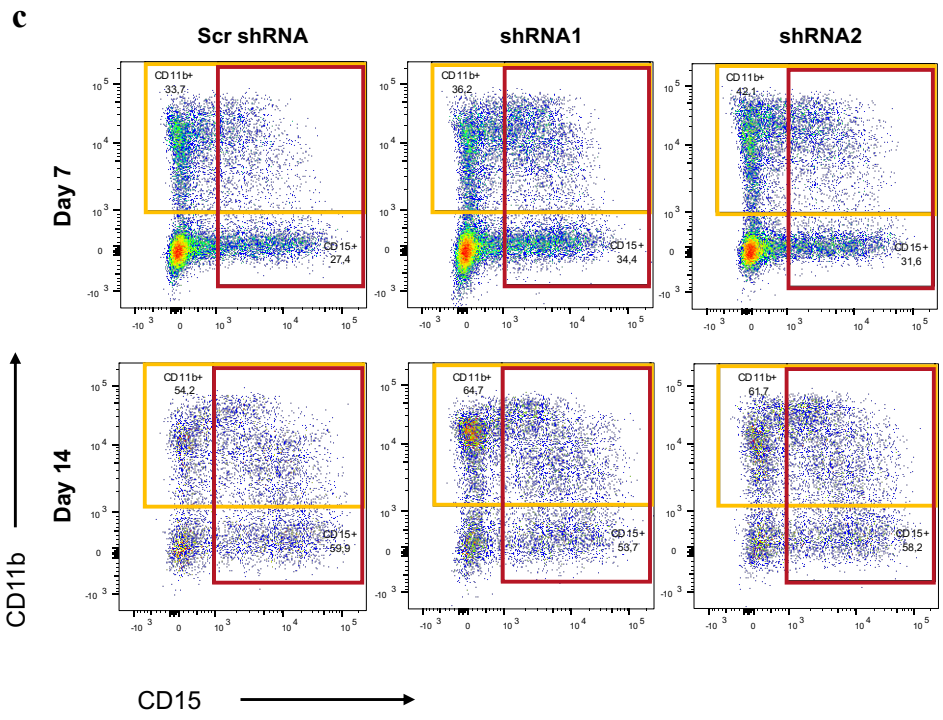

Figure 11. Knockdown of TSEN15 upregulates myeloid differentiation of HSPCs. | **a.** Quantification of CD11b marker expression at day 4, 7, 10, and 14. Data are means ± SEM of triplicate wells. | **b.** Quantification of CD15 marker expression at day 4, 7, 10, and 14. Data are means ± SEM of triplicate wells. | **c.** Representative FACS plots showing CD11b and CD15 expression on day 7 and 14. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)

4.6. TSEN15 knockdown downregulates erythroid maturation

After the differentiation assay revealed an effect of TSEN15 knockdown on the myeloid maturation, we decided to also evaluate the potential of HSPCs to differentiate into erythroid precursors. For this purpose, we used the surface marker CD71, also known as the transferrin receptor, which is expressed at high levels in erythroid precursors (Dong, Wilkes and Yang, 2011). During this experiment, the results showed that silencing of TSEN15 led to decreased expression of CD71 at day 4 and 7 of differentiation (Fig.12a and 12b). At day 4, TSEN15-targeted cells had a significantly decreased expression of CD71, reaching ~27% (shRNA1) and ~21% (shRNA2) compared with ~36% in control cells. This trend was also observed at day 7, when expression of CD71 in cells transduced with shRNA1 or shRNA2 was significantly decreased to ~18% and ~15%, respectively, compared to the control with ~22%. After 1 week of culture, the effects on the erythroid maturation counterbalanced themselves reaching an average of ~11% and 12% at day 10 and 14, respectively, in all samples. This data indicates that the knockdown of TSEN15 leads to a delay in the early erythroid differentiation.

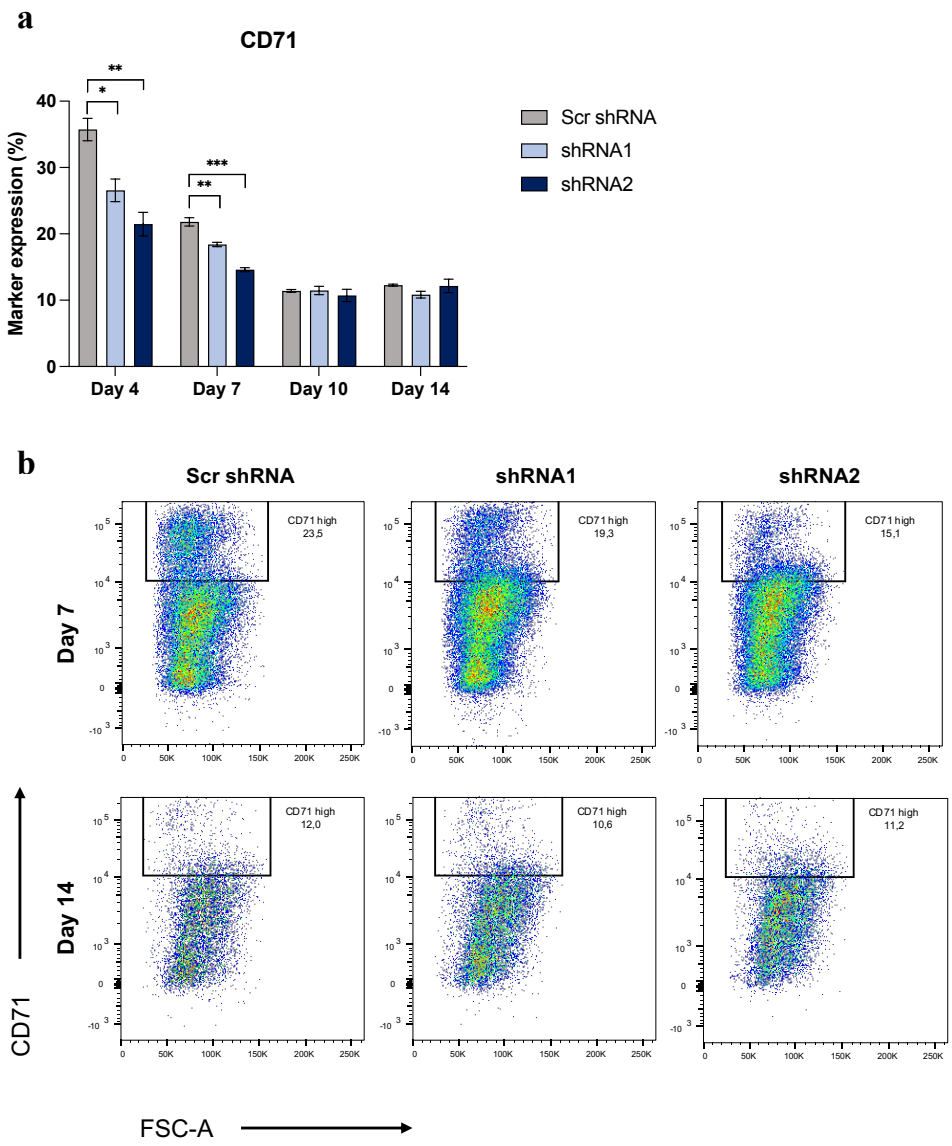

Figure 12. TSEN15 knockdown downregulates erythroid maturation. | **a.** Quantification of CD71 marker expression at day 4, 7, 10, and 14. Data are means ± SEM of triplicate wells. | **b.** Representative FACS plots showing CD71 expression on day 7 and 14. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

5. Discussion and Outlook

The remarkable ability of HSCs to continuously replenish all mature hematopoietic cells throughout life, at the same time maintaining a pool of cells with self-renewing potential, made HSCs an attractive and nowadays widely used cell type to treat patients with a wide range of diseases. However, despite the extensive research efforts to exploit the nature of HSCs, still many questions regarding the precise molecular mechanism controlling cell fate remain open. This thesis had a goal of identification of novel, genetic regulators in UCB-derived HSCs, which would influence regulation of HSCs fate in self-renewal or differentiation. We were especially interested in finding genes, whose deficiency would lead to increased *ex vivo* expansion of phenotypic HSPCs for subsequent therapeutic benefits. The work presented here brings an insight into a novel candidate gene, TSEN15, which may act as a potential negative regulator of HSPCs. In order to identify novel intrinsic regulators of HSPCs, we used an approach based on lentiviral shRNA-mediated gene silencing to target primary HSPCs. This method enables to study the function of the target gene through a stable integration of a shRNA cassette into the genome. The integration of the exogenous DNA into the cell's genome and the resulting stable expression allow to study long-term effects of the silenced gene both *in vitro* and *in vivo*. Despite the advantage of the sustained expression of the constructs in the target cells, a major problem with the use of RNAi remains the specificity of the shRNAs. Computational algorithms used for the design of the target sequences for the shRNA knockdown enable to predict shRNA potency and minimize the off-target effects. However, non-specific off-targeting is still indicated as a major bottleneck of the RNAi technology. Another issue in this context is the lentiviral delivery of shRNAs, which can result in multiple shRNA integrations in a single cell. On one hand, multiple viral integrants in a cell may be potentially harmful since they can lead to toxic downstream consequences, on the other hand, multiple shRNA integrations per host cell can also be beneficial due to a higher transcript expression, resulting in a higher gene knockdown efficiency. To keep it uniform for all tested candidates, we decided to keep the transduction efficiency between 30% and 50% to prevent multiple shRNA integrations. To counteract the problematic aspect of off-target effects within this project, we used 3 shRNAs for each target gene which target 3 differing regions in the mRNA and defined criteria when a gene should be considered as a positive hit. Namely, for further *in vitro* characterization of a potential candidate gene at least 2 shRNAs had to show a similar phenotypic effect in culture and the shRNAs had to show at least 30% knockdown of their target gene. Based on these criteria, we decided to examine the target gene TSEN15 more closely, since it complied all requirements.

In the individual validation of the selected candidate genes, TSEN15 was aside from candidate X, the only gene whose knockdown promoted a significant expansion of UCB-derived $CD34^+$ and $CD34^+CD90^+$ as well as $CD34^+EPCR^+$ cells. A closer look at the targeted cells indicates that silencing of TSEN15 may push the cell fate towards the maintenance and renewal of the immature HSCs state. Interestingly, knockdown of TSEN15 showed even a slightly higher expansion effect than candidate X, which has been found to be a negative regulator of HSPC propagation *in vitro* (unpublished data). Candidate X was identified in the course of a CRISPR/Cas9 screen, and its knockout has been shown to result in a profound increase in $CD34^+CD90^+$ cells after extended culture time. To confirm the effects observed with the CRISPR/Cas9 approach, candidate X was tested within this project using RNAi. As for TSEN15, also in case of candidate X the expansion of phenotypic HSPCs could be observed in case of 2 out of 3 used shRNAs (Fig. S1b). Gene expression analysis showed relatively low knockdown efficiencies of shRNAs targeting candidate X, ranging from ~20% to ~39% (Fig. S2a). However, this may imply that already a slight reduction in the mRNA expression levels of candidate X can expand HSPCs *ex vivo*, while a knockout intensifies the expansion phenotype. Based on these findings, it would be interesting to assess the consequence of TSEN15 knockout with CRISPR in HSPCs and see whether knockout of TSEN15 would further enhance the phenotype observed *in vitro*. Just as likely, a loss of TSEN15 could be also associated with an impairment of proliferation, thereby indicating that only a partial abrogation of TSEN15 affects expansion of HSPCs. Such a scenario is known to occur in case of the epigenetic regulator LSD1. While *in vitro* as well as *in vivo* LSD1 knockout leads to an impairment in HSPC proliferation, the partial silencing of LSD1 has been shown to promote *in vitro* propagation of HSPCs (Sprüssel *et al.*, 2012; Kerenyi *et al.*, 2013; Subramaniam *et al.*, 2020).

The fact that cells with TSEN15 knockdown exhibited maintenance of the primitive HSPCs phenotype could be an indication that silencing of TSEN15 may exert this effect by restricting hematopoietic differentiation. The evaluation of the differentiation ability of TSEN15-transduced HSPCs, however, did not show any changes regarding the ability of committed clonogenic progenitors to differentiate into erythroid and myeloid cells. Tracing of the HSPC differentiation in liquid cultures, however, revealed that shRNA-mediated inhibition of TSEN15 caused an increase in myeloid lineage marker expression that may be indicative of a myeloid skewing. Interestingly, the expression of CD71, an erythroid marker, was significantly reduced. These results indicate that TSEN15 knockdown increases myeloid differentiation but dampens erythroid maturation. Since the decrease in the expression of CD71 was observable

within the first week of culture, followed by more or less equal CD71 expression levels of control and TSEN15-targeted cells during the second week of culture, it can be assumed that TSEN15 may be involved in the regulation of the early erythroid differentiation. In order to further confirm these findings, it would be necessary to set up culture conditions, which would specifically stimulate the erythroid differentiation, and add more surface markers for the tracing of erythropoietic cells at all stages of development (e.g., CD235a, CD49d, CD233). Additionally, analysis of enucleation and hemoglobin content would shed more light on the perturbations caused by knockdown of TSEN15 during erythropoiesis. Furthermore, an analysis of TSEN15 mRNA and protein expression levels across the different populations of hematopoietic cells, including committed progenitors and mature blood cells, could also provide possible explanations for the changes observed in the erythroid and myeloid lineage commitment. Taken together, the data indicates that despite some perturbations in the myeloid and erythroid differentiation paths, TSEN15 knockdown does not prevent the differentiation of HSPCs, but rather leads to a shift towards a myeloid cell phenotype, at the same time downregulating of early erythropoiesis.

An alternative mechanism for the enhanced propagation of HSPCs observed in case of cells with TSEN15 knockdown may be an increased proliferation of the targeted cells. To confirm this assumption, it would be necessary to conduct further experiments, which would examine the cell division rate of targeted HSPCs. For instance, CFSE labelling would enable to measure cell proliferation, thereby providing information whether the cell division rate of TSEN15-targeted cells is altered compared to control cells. If cells with silenced TSEN15 would demonstrate a significantly altered cell division rate, this would suggest that their enhanced propagation is most likely due to an increased proliferation, rather than reduced differentiation. Furthermore, to gain an understanding of the mechanistic basis behind the effects observed by silencing of TSEN15, transcriptome analysis of TSEN15-deficient cells could be performed to identify signatures of genes expressed by HSPCs upon TSEN15 knockdown. It remains therefore open if TSEN15 deficiency leads, for instance, to an upregulation of genes associated with cell cycle, or apoptosis, thereby preserving an immature state of HSPCs.

Also, for a potential translation of these findings into clinical practice, it would be indispensable to assess the role of TSEN15 in a physiological context *in vivo*. Given the effects executed by TSEN15 inhibition in cultured HSPCs, it would be interesting to examine whether transplanted HSPCs with silenced TSEN15 would be able to engraft and reconstitute the various hematopoietic cell populations in immunodeficient mice. Thus, the question whether an expansion of HSPCs would be also observable *in vivo* needs to be investigated. Also, the

underlying molecular mechanism for the expansion phenotype upon TSEN15 knockdown remains to be elucidated. Since the TSEN complex plays a key role in the maturation of tRNAs, which function as bridges between mRNAs and proteins, it appears likely that perturbations in the tRNA splicing machinery, consequently leading to impaired protein synthesis, can have devastating consequences on the cells, resulting in disease conditions. Over recent years, studies have indeed pointed out that dysregulated protein synthesis affects HSC cell fate determination and can contribute to the development of hematologic malignancies, such as anemia, acute myeloid leukemia or myelodysplastic syndromes (Guzzi *et al.*, 2018, Guzzi *et al.*, 2022; Khajuria *et al.*, 2018).

Although disruptions in the splicing of precursor tRNAs and their effects on the pathogenesis of hematological malignancies remain largely unexplored, it is well known that each cell type requires an intricate balance of tRNA levels for protein synthesis and a proper cell function. Intriguingly, Singer and colleagues demonstrated a few years ago that the global protein synthesis rate in murine HSCs *in vivo* is significantly lower compared to their differentiating progeny (Signer *et al.*, 2014). The same hold true for other adult stem cell types, such as hair follicle stem cells, neural stem cells, and skeletal muscle stem cells, thereby suggesting that low rates of bulk translation are a general feature of stem cells (Llorens-Bobadilla *et al.*, 2015; Blanco *et al.*, 2016; Zismanov *et al.*, 2016). This could be expected since stem cells are metabolically quiescent, and stem cell differentiation, in contrast, is a remodeling process that demands major changes in the gene expression and cellular protein composition. Changes in the mechanisms regulating the global translation rate, including mTOR signaling, eukaryotic initiation factor 2α (eIF2α) phosphorylation, or ribosome biogenesis, have been shown to have an impact on stem cell maintenance, differentiation, and function (reviewed in Wang & Amoyel, 2022). Thus, it seems reasonable to assume that disturbances in the availability of cognate tRNAs for mRNA decoding may lead to changed mRNA translation rates and as a result to altered cell fate decisions. However, it remains an open question in which way reduced TSEN15 levels lead to upregulated proliferation of HSPCs. Disturbed tRNA biogenesis and consequently a destabilized TSEN complex may act as an HSPC-specific trigger to leave the non-cycling, quiescent state and proliferate. The fact that HSCs reside in a quiescent state characterized by a low protein synthesis rate may explain why a reduction in TSEN complex activity is well tolerated by these cells. In contrast, it has been suggested that abnormalities in tRNA biogenesis could have deleterious effects on cells with a high metabolic rate, which require a fast and localized protein synthesis, as in the case of neurons. This may also explain why defective mRNA and tRNA processing pathways often contribute to neurodegenerative

diseases (Kirchner and Ignatova, 2015; Storkebaum, 2016; Kapur, Monaghan and Ackerman, 2017; Liu, Cali and Lee, 2017). This further indicates the importance of a coordinated regulation of the tRNA biosynthesis and abundance in cells.

In summary, exploring novel regulators of HSPCs, such as TSEN15, to dissect cell fate options is not only important to expand the knowledge on stem cell regulation, but it is also the key for the future of innovative stem cell gene therapies. The search for feasible stem cell expansion approaches is based on a strong medical demand since the clinical application of expanded HSCs would circumvent the limitation of low stem cell doses in UCB units. Overcoming this problematic aspect associated with UCB as a practical stem cell source would enable the use of better suited HLA-matched UCB units for patients who require an allogenic HSCTs. Even though there is a long way to go towards the implementation of findings made at the laboratory bench into clinical applications, the work presented here gives a small insight into a potential negative regulator of HSPC expansion, TSEN15, whose inhibition supports the expansion of phenotypic and functional HSPCs in culture without affecting the overall differentiation potential of HSPCs. However, since in general little is known at the moment about the cellular functions of TSEN15 and its involvement in pathological conditions, more research will be required to gather more information concerning the role of TSEN15 in hematopoiesis, but also in a broader biological context.

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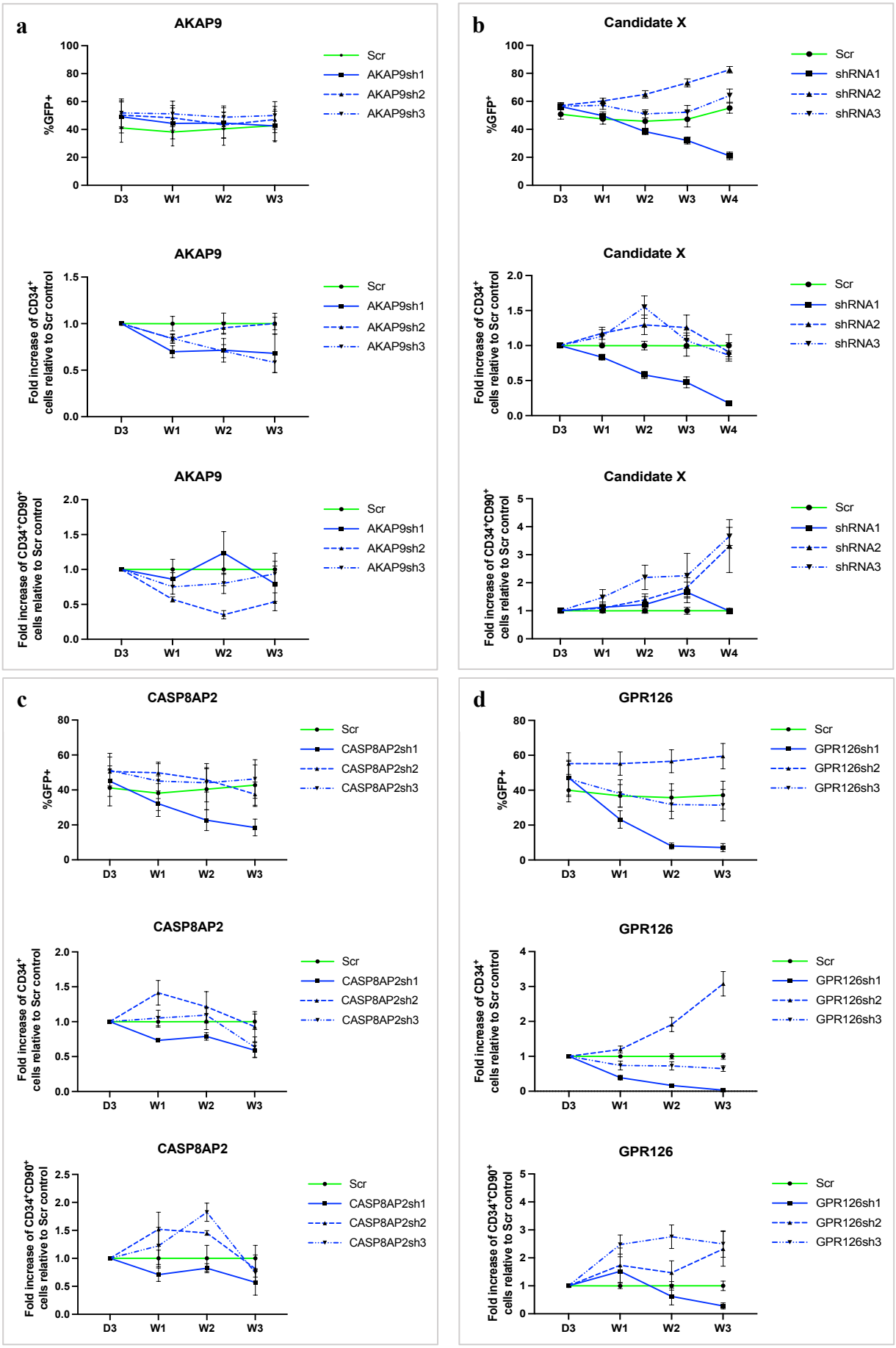
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Candidate X
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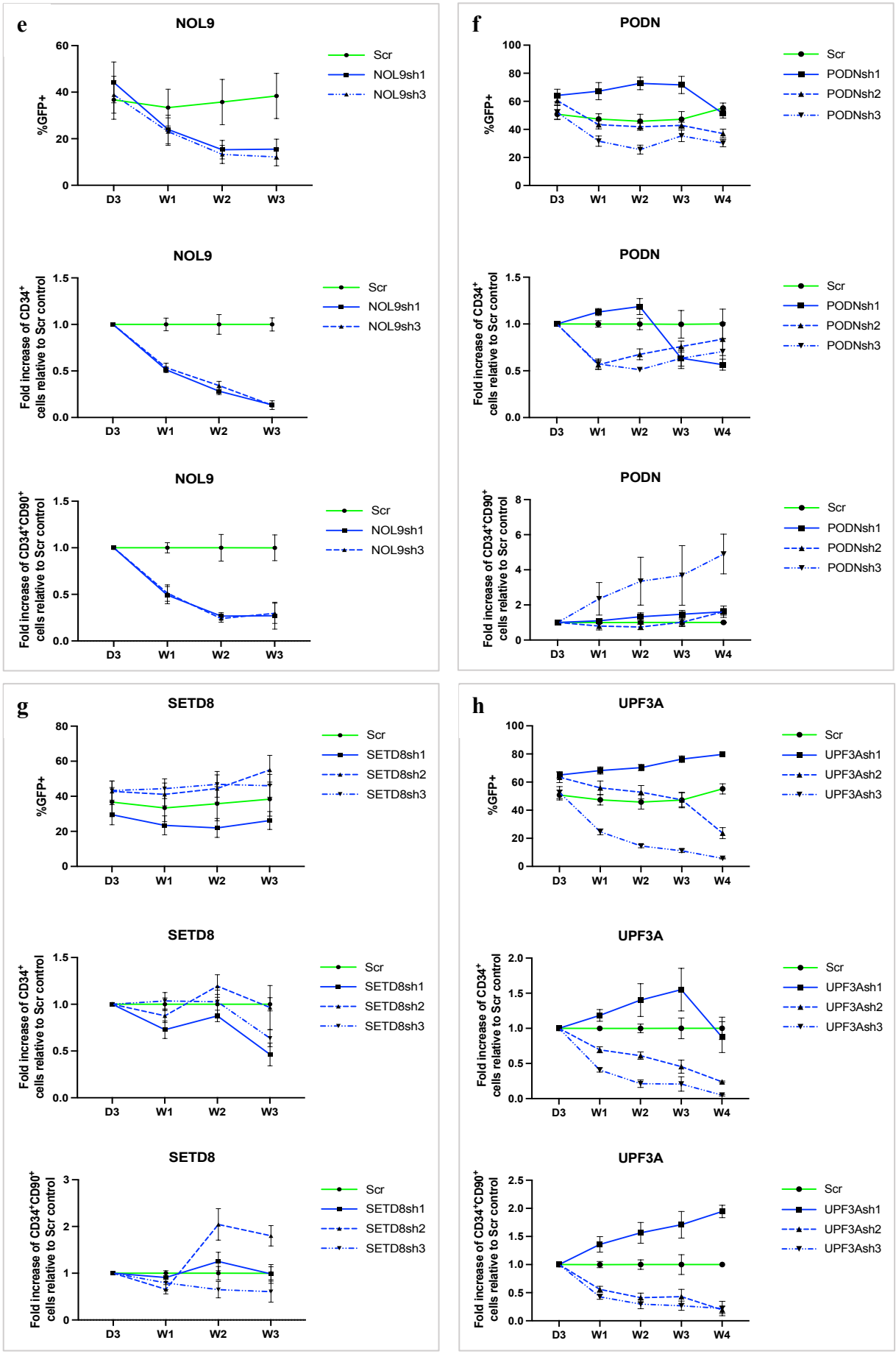
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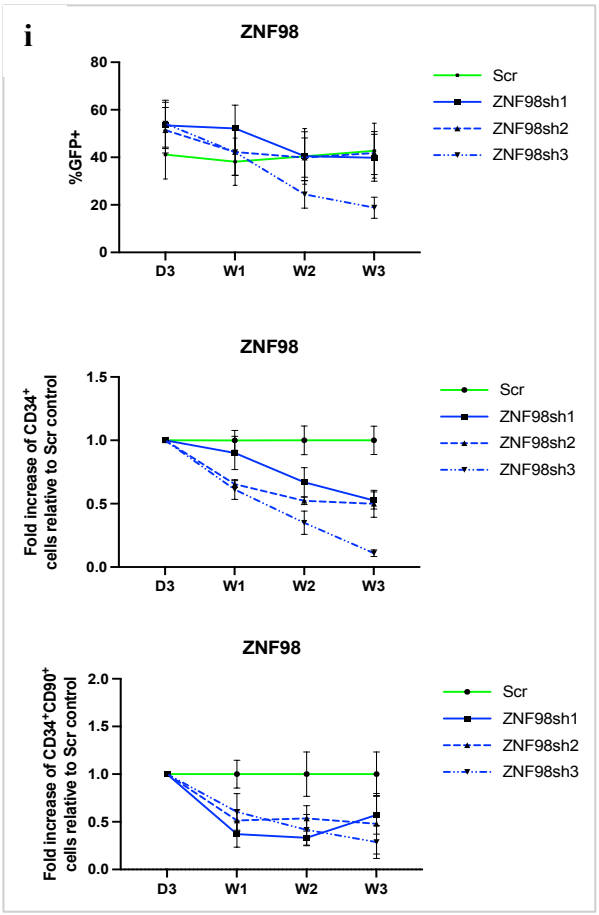

Figure S1. Expansion of the GFP$^+$CD34$^+$ and GFP$^+$CD34$^+$CD90$^+$ cell numbers over a period of 3 or 4 weeks for **a.** AKAP9, **b.** Candidate X, **c.** CASP8AP2, **d.** GPR126, **e.** NOL9, **f.** PODN, **g.** SETD8, **h.** UPF3A, **i.** ZNF98 gene compared to scrambled control. Data from at least 2 independent experiments with 3 replicates each. | D, Day; W, Week, Scr, Scrambled.

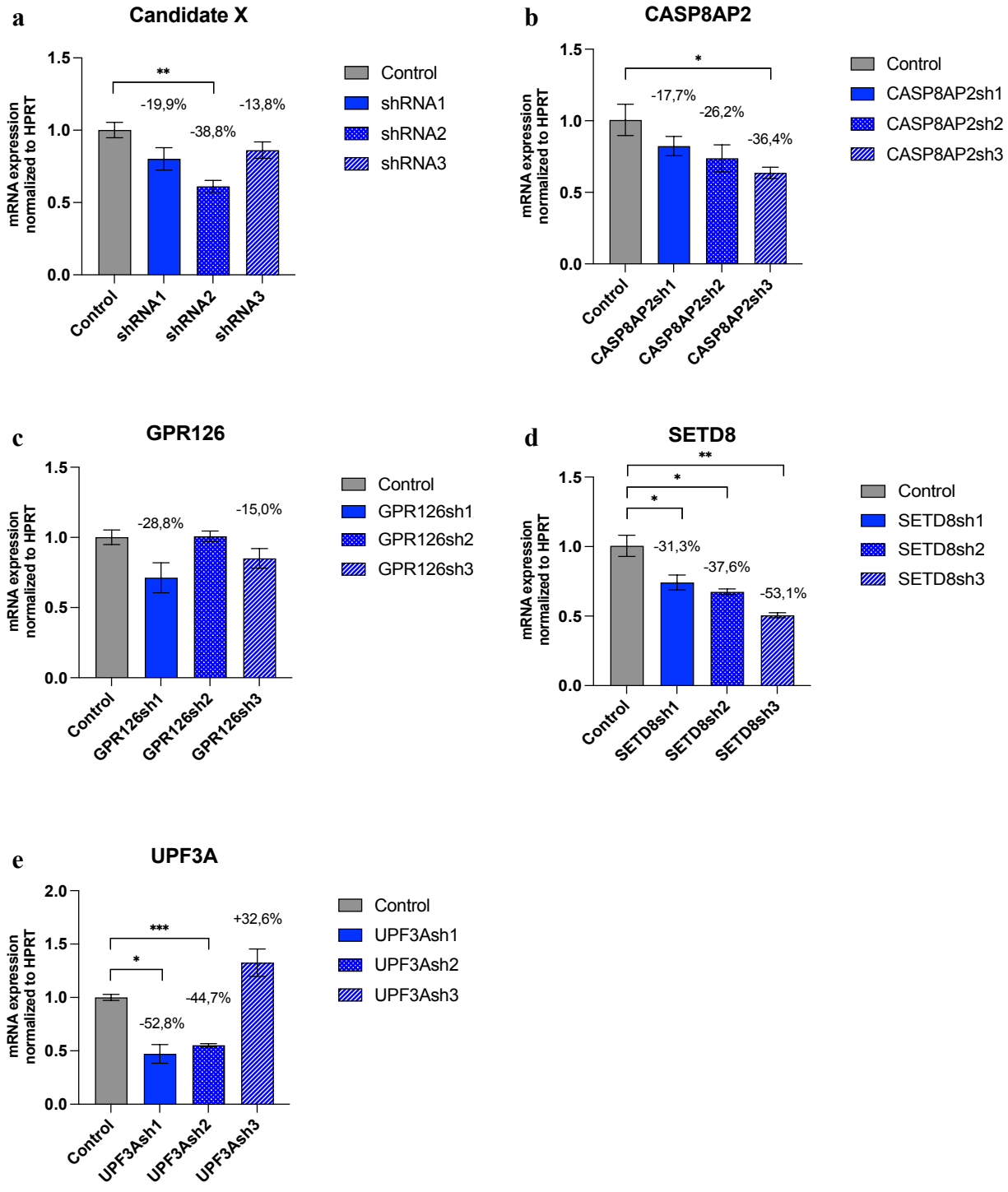

Figure S2. Knockdown efficiency of shRNAs targeting **a.** Candidate X, **b.** CASP8AP2, **c.** GPR126, **d.** SETD8, **e.** UPF3A as measured by qPCR. Data from one experiment with 9 replicates. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)