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Targeting the hematopoietic stem cell to correct osteopetrosis

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Targeting the Hematopoietic Stem Cell to Correct Osteopetrosis

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To my family

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Abbreviations

ADA	adenosine-deaminase
AGM	aorta-gonads-mesonephros
Ang-1	angiopoietin 1
BM	bone marrow
CFU-S	colony-forming-unit spleen
CGD	chronic granulomatous disease
EF1-α	Elongation factor 1- alpha
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FL	fetal liver
GFP	green fluorescent protein
GVDH	graft-verus-host-disease
HIV-1	human immunodeficiency virus 1
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
IL	interleukin
IMO	infantile malignant osteopetrosis
IP	intraperitoneal
IV	intravenous
LAM	linear amplification–mediated
Lin⁻	lineage negative
LMO2	LIM domain only 2 (rhombotin-like 1)
LTR	long terminal repeat
M-CSF	macrophage colony-stimulating factor
MSC	mesenchymal stem cell
NK	natural killer
MUD	matched unrelated donor
NSG	NOD/LtSz-scid IL-2R gamma null
OB	osteoblast

OC	osteoclast
OPN	osteopontin
OSTM1	osteopetrosis-associated transmembrane protein 1
PB	peripheral blood
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PGK	phosphoglycerate kinase
PIC	pre-integration complex
RANK	receptor activator of nuclear factor κ B
RANKL	receptor activator of nuclear factor κ B ligand
RRE	rev responsive element
RT	reverse transcriptase
Sca-1	stem cell antigen 1
SCF	stem cell factor
SCID	severe combined immunodeficiency
SCT	stem cell transplantation
SDF-1	stromal derived factor 1
SFFV	spleen focus forming virus
SLAM	signaling lymphocyte activation marker
SNS	sympathetic nerve system
TBI	total body irradiation
TCIRG1	T cell immune regulator 1
TGF-β	transforming growth factor-β
VCAM	vascular cell adhesion molecule
VCM	virus containing medium
VLA-4	very late antigen 4
VSV-G	vesicular stomatitis virus glycoprotein
WT	wild type
ZFN	zinc-finger nuclease

Articles and manuscripts included in this thesis

1. Askmyr M*, Holmberg J*, **Flores C**, Ehinger M, Hjalt T, Richter J. Low-dose busulphan conditioning and neonatal stem cell transplantation preserves vision and restores hematopoiesis in severe murine osteopetrosis. Experimental Hematology, 2009; 37, 302–308.
* These authors contributed equally to this work.

2. **Flores C**, Vries TJ , Moscatelli I , Askmyr M, Schoenmaker T, Langenbach G EJ, Ehinger M, Everts V, Richter J. Nonablative Neonatal Bone Marrow Transplantation Rapidly Reverses Severe Murine Osteopetrosis Despite Low-Level Engraftment and Lack of Selective Expansion of the Osteoclastic Lineage. Journal of Bone and Mineral Research, 2010; 25, 9, 2069–2077.

3. Moscatelli I*, Thudium CS*, **Flores C**, Askmyr M, Schulz A, Porras O, Karsdal MA, Fasth A, Villa A, Henriksen K, Richter J. TCIRG1 Lentiviral Gene Transfer to Peripheral Blood CD34+ restores Osteoclast Function in Infantile Malignant Osteopetrosis. Molecular Therapy.
* These authors contributed equally to this work.

4. **Flores C*,** Thudium C*, Moscatelli I*, Gudmann CN, Henriksen K, Richter J. Osteoclast mediated bone resorption is not essential for hematopoietic stem cell maintenance in adult mice.
* These authors contributed equally to this work.

Introduction

The endless ability of the hematopoietic stem cell (HSC) to generate all mature cells in the blood makes it an attractive candidate when developing new therapies. Hematopoietic stem cell transplantation is today an established treatment for hematopoietic malignancies and genetic disorders of the blood and immune system. Still, extensive research is necessary to face the challenges of HSC expansion *in vitro* and tackling the complications of graft versus host disease (GVHD) when performing allogeneic stem cell transplantation. Using hematopoietic stem cell targeted gene therapy normal function can be restored in patient cells by correcting or inserting a mutated or missing gene. In the gene therapy setting, the patient´s own cells can be used without the need of finding a suitable donor. The genetically modified HSCs and their progeny will last throughout life and thereby potentially cure the disorder. Producing pluripotent stem cells by reprogramming adult somatic patient cells would be yet another way of providing the stem cells needed. Although there is still a long way to go before induced pluripotent stem cells (iPSCs) can be used in the clinic, this groundbreaking achievement is of great importance for stem cell research, offering the possibility of producing any desired cell type in the body including HSCs. The development of efficient and safe gene therapy and regenerative medicine offers great promise for the future and these treatment modalities have the potential to rescue patients with no alternative treatment option.

This thesis focuses on developing stem cell targeted gene therapy for the severe hereditary disorder Infantile Malignant Osteopetrosis (IMO) as well as increasing the understanding of how the genetic defect present in IMO affects the HSCs and hematopoiesis in general.

The Hematopoietic Stem Cell

HSCs represent a rare population of cells that can be defined by their capacity for long-term self-renewal and multi-lineage differentiation. With these characteristics the HSC supplies the human body with billions of new blood cells every day, ensuring a continuous, lifelong hematopoiesis (Ogawa 1993; Ema, Sudo et al. 2005). The HSCs are primarily located in the bone marrow and are mainly quiescent cells in adults. Still they do enter the cell cycle regularly and a small percentage (3-5%) is always found in active cell cycle contributing to the hematopoietic output during steady state (Bradford, Williams et al. 1997; Cheshier, Morrison et al. 1999). By rarely entering the cell cycle, keeping a low proliferative state, the cells are protected from DNA damage (Yahata, Takanashi et al. 2011). It has been shown that there is a rare population of HSCs, called dormant cells that are even more quiescent and divide only every 4-5 months. These cells serve as a back-up pool of cells needed for stress situations (Wilson, Laurenti et al. 2008). The regulation of HSC fate decision continues throughout the life of the cell and the HSC can either self-renew through symmetric division generating two identical progenies or through asymmetric division yielding two daughter cells with different fates. The cell can also commit to differentiate to a specific lineage, migrate to a new location or undergo apoptosis (Wagers, Christensen et al. 2002). The determination of HSC fate is most likely a combination of stochastic intrinsic events during HSC division and deterministic events due to extracellular signals from the HSC niche (Morrison and Weissman 1994; Enver 1998; Metcalf 1998; Ogawa 1999).

The hematopoietic development is a hierarchical process and at the top of the hematopoietic hierarchy we find a few slow cycling HSCs, called long-term HSCs (LT-HSCs). These are the true stem cells with the ability to self renew indefinitely. They are followed by the short-term HSCs (ST-HSCs) that are multipotent cells but have a limited self-renewal capacity and can only contribute to hematopoiesis transiently (Morrison, Wandycz et al. 1997; Yang, Bryder et al. 2005). Further downstream the cells start to differentiate and become more lineage-restricted progenitors while losing their self-renewal capacity. At the

bottom of the hierarchy we find all the mature blood cells (Orkin 2000; Weissman 2000).
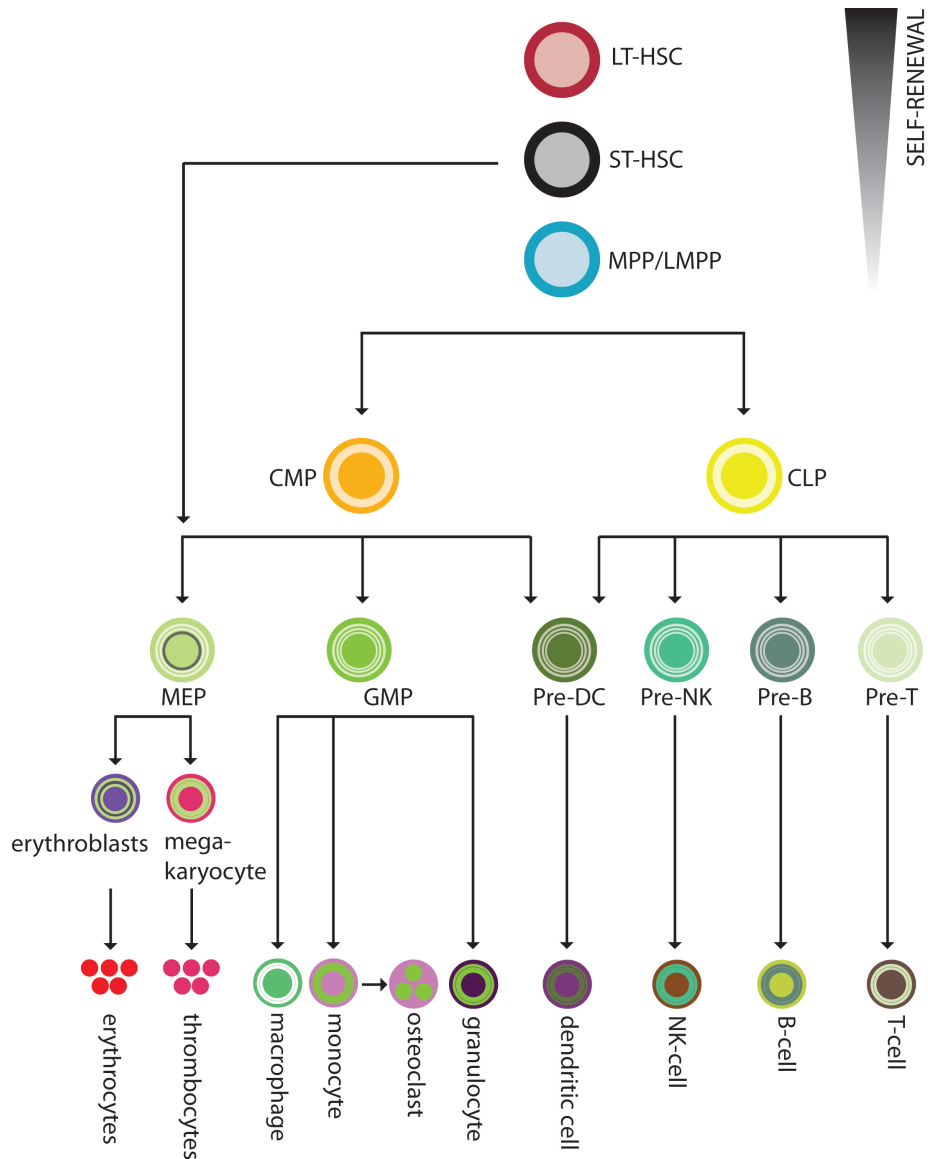
Figure 1. An overview of the hematopoietic hierarchy
The picture is a simplified illustration of hematopoiesis, which is a complex system with no current consensus on the detailed structure. LT-HSC (long term HSC), ST-HSC (short-term HSC), MPP/LMPP (multipotent progenitor/lymphoid-primed multipotent progenitor), CMP (common myeloid progenitor), CLP (common lymphoid progenitor), MEP (megakaryocyte-erythrocyte progenitor), GMP (granulocyte-monocyte progenitor), Pre-DC (pre-dendritic cell), Pre-NK (pre-natural killer cell), Pre-B (pre-B lymphocyte) and Pre-T (pre-T lymphocyte).

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Hematopoietic cells can be identified and isolated using Fluorescence-activated cell sorting (FACS). All blood cells express specific cell-surface antigens that can be labeled with antibodies conjugated to fluorescent markers. The HSCs represent an extremely rare population of cells, and because there is no known individual marker for the HSC it is possible to identify more purified populations of HSCs and progenitors by combining these different antibodies.

Murine HSCs are found within a population phenotypically characterized by the lack of specific lineage surface markers and by expressing stem cell antigen-1 (Sca-1) and c-kit markers ($L^-S^+K^+$) (Muller-Sieburg, Whitlock et al. 1986; Spangrude, Heimfeld et al. 1988; Okada, Nakauchi et al. 1991). LT-HSCs can further be purified by the lack of CD34 and Flt3 surface marker expression (LSK $CD34^-$ $Flt3^-$) (Osawa, Hanada et al. 1996) (Adolfsson, Borge et al. 2001). Another way of identifying these cells is by the expression of signaling lymphocyte activation marker (SLAM) receptors, $CD150^+CD244^-CD48^-$ (Kiel, Yilmaz et al. 2005). The human LT-HSCs on the other hand, express the CD34 surface marker and can be further purified by the expression of CD38, and expression of CD90 and CD49f ($CD34^+CD38^-CD90^+CD45RA^-CD49f^+$) (Notta, Doulatov et al. 2011). Besides staining cell surface markers, the HSCs can also be isolated by staining with different dyes that bind to the mitochondria (Rhodamine 123), DNA (Hoechst 33342), or RNA (Pyronin Y) of the cells. The HSCs efflux these dyes through ATP binding cassette (ABC) transporters and HSCs can therefore be identified by containing low levels of dye (Zhou, Schuetz et al. 2001).

Ultimately, it is important to remember that the HSCs are defined by their function and the only way of confirming that the cells have the correct stem cell properties is by functionally testing them in transplantation assays. True LT-HSCs can be studied by long-term transplantation assays in mice, and analyzed for multi-lineage reconstitution of donor cells after more than 12 weeks. The cells can further be transplanted into secondary and tertiary recipients to confirm continuous self-renewal capacity. Another transplantation assay is competitive transplantation, where stem cells compete for their niches and thereby it is possible to measure the function of HSCs (Purton and Scadden 2007).

Development of the HSC

In early embryogenesis, the blastocyst is reorganized to form the three germ layers ectoderm, endoderm and mesoderm, a process referred to as gastrulation. Hematopoietic cells arise from the mesoderm germ layer, more precisely from the hemangioblast formed in the yolk sac at embryonic day 7.5 (E7.5). The hemangioblast serves as a common mesodermal precursor for hematopoietic and endothelial cells and provides the embryo with nucleated erythrocytes, known as primitive hematopoietic precursors, expressing fetal haemoglobin (Moore and Metcalf 1970; Choi, Kennedy et al. 1998; Mikkola, Fujiwara et al. 2003; Cumano and Godin 2007). Primitive hematopoiesis is essential for providing the embryo with oxygen but it is not believed to comprise any true HSCs (Cumano, Candido Ferraz et al. 2001). These true HSCs appear at E10 in the aorta-gonads-mesonephros (AGM) region in the embryonic body and in the placenta (Gekas, Dieterlen-Lievre et al. 2005) and support the definitive hematopoiesis, providing persistent long-term hematopoiesis (Medvinsky and Dzierzak 1996). As the cells mature they enter the blood circulation and migrate to the fetal liver that becomes the main hematopoietic organ from E11. In the fetal liver the HSCs undergo a massive expansion to meet the requirements of the growing embryo (Morrison, Hemmati et al. 1995; Ema and Nakauchi 2000; Mikkola and Orkin 2006). Finally at E16.5 the HSCs begin to relocate to the BM, at the same developmental stage as the trabecular bone is formed, which remains the main site for hematopoiesis throughout life (Nilsson, Johnston et al. 2001; Grassinger, Haylock et al. 2010).

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The HSC Niche

The HSC niche can be described as a spatial residence comprising many different cell types that create the proper microenvironment needed for HSC maintenance (Schofield 1978; Kiel, Yilmaz et al. 2005; Lo Celso, Fleming et al. 2009; Xie, Yin et al. 2009). The specialized microenvironment is located within the BM close to the bone, and is thought to be hypoxic. The low oxygen level protects HSCs from reactive oxygen species (ROS), which can induce cycling of quiescent HSCs, ultimately leading to exhaustion (Ito, Hirao et al. 2006; Parmar, Mauch et al. 2007; Kubota, Takubo et al. 2008). Two independent anatomical niches have been proposed, the endosteal niche also known as the osteoblastic niche and the vascular niche (Ehninger and Trumpp 2011). Whether both are the true stem cell niches or not and how they are regulated remains controversial (Lo Celso, Fleming et al. 2009; Xie, Yin et al. 2009; Winkler, Barbier et al. 2010).

Niche regulation of HSCs

The HSCs interaction with their microenvironment is critical when maintaining normal hematopoiesis and their specific fate is determined through complex, bidirectional interactions with various cell types and stromal cell components. In the osteoblastic niche the HSCs reside in close contact with a subset of osteoblast progenitors through adhesion molecules e.g. N-cadherin, which play a key role in the HSC regulation (Nakamura, Arai et al. 2010; Raaijmakers, Mukherjee et al. 2010). The importance of the osteoblast (OB) in HSC maintenance was first shown in 2003, in two independent studies. In one study, the murine BMP receptor 1a was deleted which resulted in increased number of spindle shaped osteoblastic cells expressing N-cadherin and subsequently increased numbers of HSCs (Zhang, Niu et al. 2003). In the second study, parathyroid hormone signaling was constitutively activated through expression of the osteoblast specific PTH/PTHrP receptor (PPR) in a transgenic mouse model, resulting in elevated Jagged1 expression and increased Notch signaling, also leading to increased number of HSCs (Calvi, Adams et al. 2003). Further, the OB expresses Angiopoietin 1,

which binds to the Tie-2 receptor expressed on HSCs, keeping them in a quiescent state (Zhang, Niu et al. 2003; Arai, Hirao et al. 2004). OBs also secrete osteopontin (OPN), a matrix glycoprotein that functions as a negative regulator of HSCs (Nilsson, Johnston et al. 2005; Stier, Ko et al. 2005).

Sinusoidal endothelial cells have been proposed as an alternative HSC niche, the vascular niche, and the cells seem to be crucial for engraftment of HSCs after irradiation (Hooper, Butler et al. 2009). Yet another important regulator is the stem cell factor (SCF), which contributes to the self-renewal and maintenance of HSCs in vivo by binding to the c-kit receptor expressed on HSCs (Ding, Saunders et al. 2012). Mice with a partial loss of the kit function (kit [W41/41]) have a 2.4-fold reduction of LT-HSC (Thoren, Liuba et al. 2008). The SCF is also important in the migration of the HSCs and, in conjunction with the chemoattractant stromal derived factor 1 (SDF-1 or CXCL12), regulation of the HSCs mobilization from the BM niche into the circulation (Lapidot and Kollet 2002; Méndez-Ferrer, Lucas et al. 2008). Other cell surface adhesion molecules important in migration, especially in homing, are the β1 integrin Very Late Antigen 4 (VLA-4) and the ligand Vascular Cell Adhesion Molecule (VCAM) (Papayannopoulou, Craddock et al. 1995).

The bone resorbing osteoclast (OC) has also been shown to play a crucial role in the niche regulation. The OCs are formed from circulating monocytes, in the presence of the cytokines macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL), secreted by OBs and stromal cells (Udagawa, Takahashi et al. 1990; Yoshida, Hayashi et al. 1990; Li, Sarosi et al. 2000). OCs and OBs are essential for the formation of the osteoblastic niche during ontogeny (Chan, Chen et al. 2009; Mansour, Abou-Ezzi et al. 2012). Furthermore, the OCs promote mobilization of HSCs and progenitors from the niche to the circulation by cathepsin K-mediated cleavage of CXCL 12 (Kollet, Dar et al. 2006). HSCs express calcium-sensing receptors (CaR) and it has also been demonstrated that mice lacking CaR have reduced numbers of HSCs in the BM, indicating that the calcium, released following OC-mediated bone resorption, is crucial for the correct localization of HSCs (Adams, Chabner et al. 2006; Jeanson and Scadden 2010; Lam, Cunningham et al. 2011).

Other important cell types involved in mobilization are mesenchymal cells, e.g. fibroblast-like reticular cells and Nestin[+] mesenchymal stem cells (MSC) that express high levels of SDF-1/CXCL12 (Sugiyama, Kohara et al. 2006; Mendez-Ferrer, Michurina et al. 2010). In addition, macrophages have been proposed to

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inhibit mobilization of HSCs (Winkler, Sims et al. 2010; Chow, Lucas et al. 2011).

The sympathetic nerve system (SNS) also plays an important role in the niche regulation. Through homeostatic circadian rhythms, the SNS controls the constant remodelling of the niche through the control of bone cells and the mobilization of HSCs (Katayama, Battista et al. 2006; Takeda 2008). Other extrinsic regulators in the niche include transforming growth factor-β (TGF-β) (Langer, Henckaerts et al. 2004; Karlsson, Blank et al. 2007; Yamazaki, Ema et al. 2011) and Wnts, which have been preserved throughout development and play an important role in maintaining and regulating the HSC (Reya, Duncan et al. 2003; Duncan, Rattis et al. 2005; Trompouki, Bowman et al. 2011). The extrinsic signals from the niche will most likely influence the intrinsic regulation of the HSCs and ultimately determine the fate of the cells.
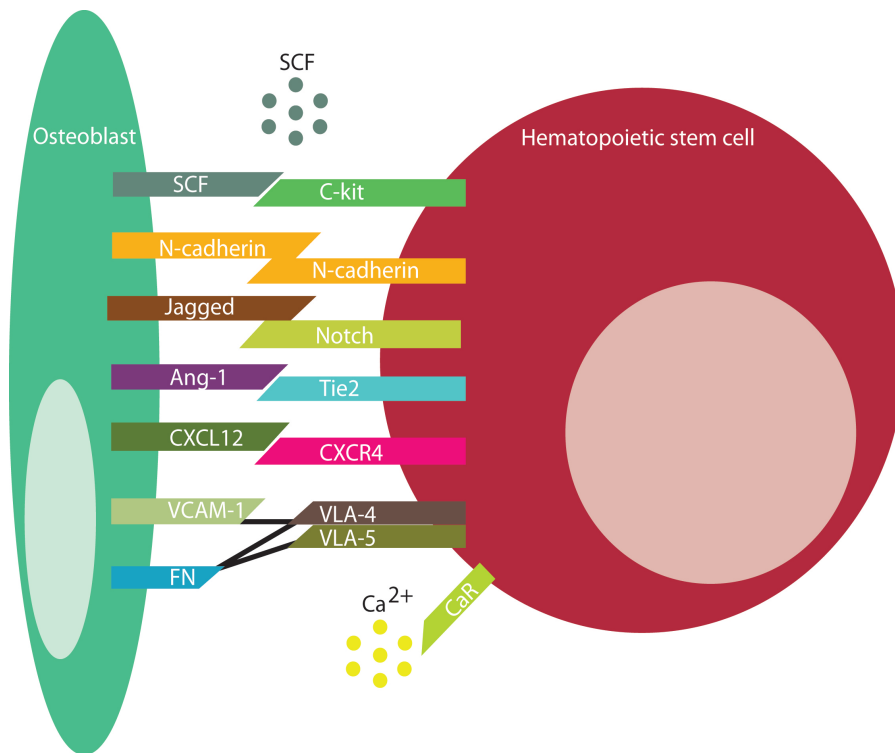

Figure 2. Quiescent HSC in the osteoblastic niche
The HSC is attached to the osteoblast by OB adhesive molecules e.g. N-cadherin and VLA-4/5 with VCAM and fibronectin and by providing the appropriate signals e.g. Jagged and Ang-1, the HSC is maintained quiescent. SCF, SDF-1/CXCL12 and Ca-ions involved in the mobilization of HSCs are also shown.

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HSC Transplantation

After the nuclear bombing in Japan 1945 researchers observed that the BM is sensitive to radiation and these findings resulted in the discovery that by transplanting BM cells, fatal outcome due to lethal radiation can be prevented (Lorenz, Uphoff et al. 1951; Ford, Hamerton et al. 1956). The first human BM transplantations were successfully performed in 1959 although it took another couple of years before the importance of human leukocyte antigen (HLA)-matched donors was understood (Thomas, Lochte et al. 1959; Thomas 2000). In 1961 two pioneers of stem cell research, Till and McCulloch, showed that transplanted BM cells were able to home to the spleen and form multi-lineage forming colonies, named spleen colony forming units (CFU-S) in irradiated mice (Till and McCulloch 1961). The colonies were also capable of producing all blood lineages when transplanted into secondary recipients. This led to the idea of a stem cell, a cell capable of self-renewal as well as differentiating into all other cells in the blood (Becker, McCulloch et al. 1963; Siminovitch, McCulloch et al. 1963). Since then HSC transplantation (HSCT) has become an established method in the clinic for the treatment of hematopoietic malignancies and genetic diseases.

HSCT can be performed by transplanting the patient´s own cells, called autologous HSCT, or by transplanting stem cells from a HLA-matched donor, called allogeneic HSCT. HLAs are expressed by the white blood cells and are specific for each individual so the immune system uses the HLAs to distinguish self from non-self. Graft-versus-host-disease (GVDH) is a serious complication after allogeneic HSCT and is due to the attack of transplanted donor cells that recognize the recipient´s HLAs as non-self (Copelan 2006).

Options for patients lacking an acceptable HLA-matched donor (sibling or register), is using haplo-identical stem cells from one of the parents (or children) or using cord blood cells (CBs). CB is a promising source of stem cells but the limitations in HSCT is the low quantity of stem cells obtained. A major interest in stem cell research is to successfully expand stem cells ex vivo and although some progress has been achieved, extensive research is still needed before these findings can be more generally applied in the clinic (Boitano, Wang et al. 2010; Delaney, Heimfeld et al. 2010; Himburg, Muramoto et al. 2010).

Different conditioning regimens are most often used before transplantation and this varies according to the type of transplantation. Radiation therapy or combinations of chemotherapy can be used. Cyclophosphamide and busulfan are two non-specific alkylating agents commonly used, that bind to DNA and inhibit DNA replication and induce cell death.

For autologous HSCT, the purpose of the conditioning is to destroy malignant cells and provide space in the BM for transplanted cells, however for allogeneic transplants, the conditioning is also needed for immunosuppression of the recipient to avoid graft rejection (Copelan 2006).

HSC based gene therapy

The basic principles of HSC based gene therapy is to transfer genetic information to restore or modify the function of a cell. In many monogenetic hematological disorders gene therapy potentially offers an alternative treatment modality when no matching donors are available.

Several primary immunodeficiencies have been treated with HSC targeted gene therapy using gammaretroviral vectors, e.g. X-linked SCID (Cavazzana-Calvo, Hacein-Bey et al. 2000; Gaspar, Parsley et al. 2004), adenosine deaminase-SCID (ADA-SCID) (Aiuti, Slavin et al. 2002) and also chronic granulomatous disease (CGD) (Ott, Schmidt et al. 2006). A disadvantage of the gammaretroviral vector is that cell division is required for transduction and integration. Lentiviral vectors on the other hand, are able to transduce both dividing and non-dividing cells eliminating the requirement of stimulatory cytokines for cell division. (Naldini, Blomer et al. 1996; Case, Price et al. 1999; Miyoshi, Smith et al. 1999). However, in our protocol we use pre-stimulation since this leads to increased transduction efficiency. Lentiviral vectors are significantly less prone to insert in proto-oncogenes compared to gammaretroviral vectors, giving them a better safety profile (Cattoglio, Facchini et al. 2007; Neschadim, McCart et al. 2007).
Diseases where successful gene transfer to human HSCs has been achieved using a lentiviral vector are CGD (Naumann, De Ravin et al. 2007), Wiskott-Aldrich Syndrome (Martin, Toscano et al. 2005; Charrier, Dupre et al. 2007) and X-Linked Adrenoleukodystrophy (Cartier, Hacein-Bey-Abina et al. 2009).

Lentivirus

Lentiviral vectors are based on the genetics of lentivirus and are a subfamily of the retrovirus family. Members of the lentivirus are the human immunodeficiency virus 1 (HIV-1), simian immunodeficiency virus (SIV), bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV). Here I will focus on HIV-1, which is the origin of the vector used in this thesis. Characteristic for the retroviruses is the reverse transcriptase (RT)

enzyme required for producing DNA from the two single stranded RNA copies that make up their genome. This is a necessity for the virus to be able to replicate, since the only way of doing so is by integrating the reversed transcribed DNA into the genome of the host cell and using its machinery for transcription. Another advantage with the HIV-1 virus is that the pre-integration complex (PIC) can remain stable for a long time, waiting for the optimal opportunity to integrate into the host cell genome, a quality that enables the vector to transduce quiescent cells (Bukrinsky, Sharova et al. 1992; De Rijck, Vandekerckhove et al. 2007).
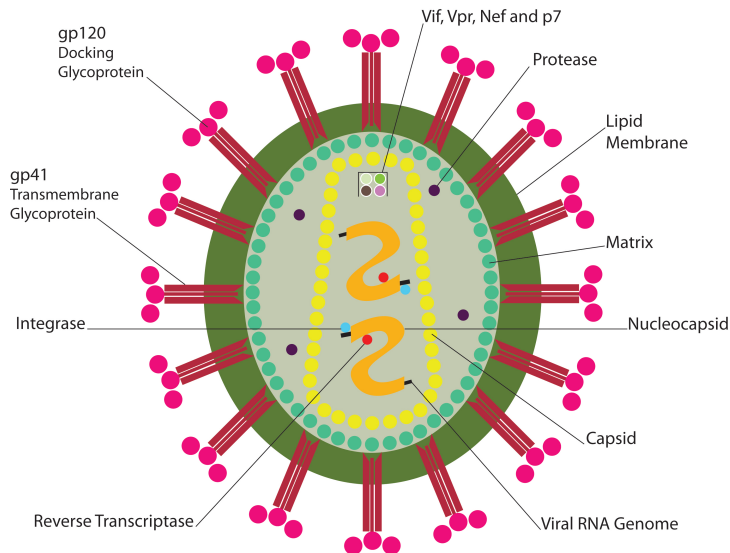
Figure 3. Wild-type HIV-1 virus

The wild-type HIV consists of an envelope that surrounds a nucleocapsid that contains the viral genome. The envelope is derived from the plasma membrane of the host cell as well as the glycoproteins (gp120 and gp41) encoded by the env-gene. The capsid and nucleocapsid are made of proteins encoded by the gag gene e.g. p17 (matrix), p24 (capsid), p6/7 (nucleocapsid) and within the nucleocapsid are the pol encoded RT, protease and integrase, required for the synthesis and integration of viral DNA into host cells. The HIV-1 genome also contains six additional accessory genes: rev, tat, nef, vpr, vpu and vif. Rev and tat are two important regulators of gene expression and the four remaining genes nef, vpr, vpu and vif are important for efficient spreading of the virus.

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Lentiviral vector construction

The natural capability of retroviruses to integrate into a cell and deliver genetic material is utilized when making viral vectors. By removing most of the viral genes, the vector is made replication-incompetent and can therefore be used as a delivering device of the desired gene to create permanent gene transfer.

The general current method when producing a lentiviral vector is to transiently transfect a producer cell line (often 293T) with three or four plasmids that together encode for the genes necessary for vector assembly and function. These required HIV-1 genes have been separated in cis- and trans-acting sequences. The vector construct contains cis expressing genes that will be integrated into the host genome e.g. the transgene, packaging sequences, and rev-response element (RRE) necessary for mRNA nuclear export to the cytoplasm. It also contains the central polypurine tract (cPPT) to improve the PIC nuclear import efficiency and the woodchuck post-trancriptional regulatory element (wPRE) known to increase transgene expression (Schambach, Bohne et al. 2006). The remaining genes, expressed in trans, only need to be expressed in a producer cell line. These genes are separated into the three plasmids, containing the gag/pol gene and the rev gene and the envelope gene. This four-plasmid system is referred to as the third generation lentiviral vector and differs from the second-generation vector by lacking the tat gene (Dull, Zufferey et al. 1998). Furthermore, in both the second- and third-generation vector, the 3´and 5´UTR have been deleted and are referred to as self-inactivating (SIN) vectors. They contain an internal promoter of choice instead, which minimizes the risk of insertional activation of neighboring genes (Miyoshi, Blomer et al. 1998; Zufferey, Dull et al. 1998).
The envelope gene encodes the glycoproteins expressed on the vector and a commonly used envelope protein is the vesicular stomatitis virus glycoprotein (VSV-G) because of its wide range of cell specificity (Ory, Neugeboren et al. 1996). The envelope glycoproteins can be replaced with those of other viruses in order for the vector to transduce specific target cells, a process termed pseudotyping.
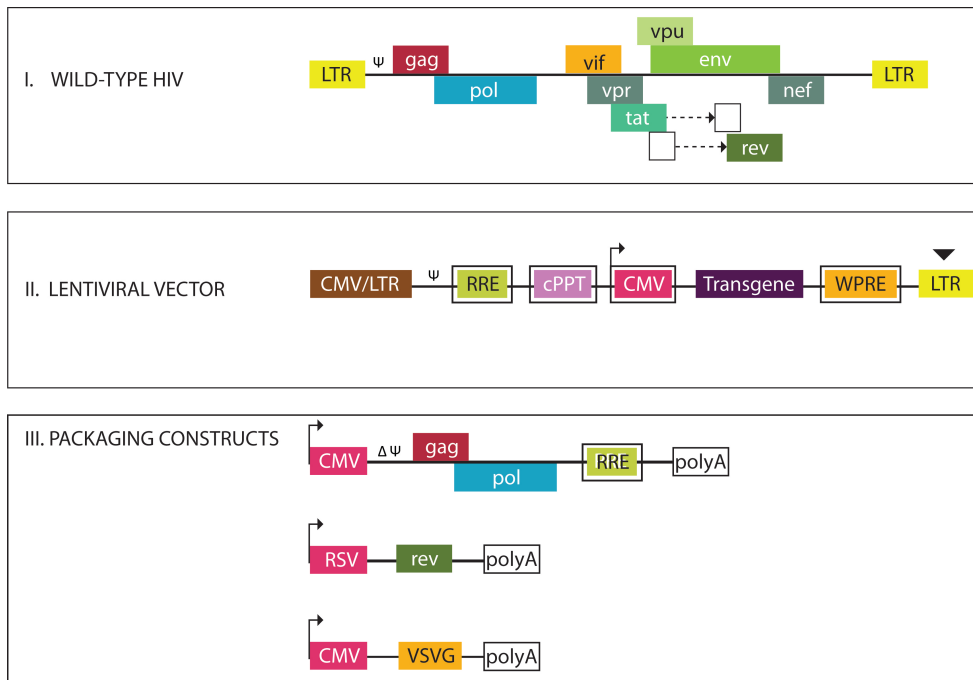
Figure 4. Lentiviral vector construction

Challenges in HSC targeted gene therapy

More than a decade has passed since the first clinical gene therapy trials demonstrated successful restoration of the immune system for children with primary immunodeficiencies. Unfortunately, 5 out of 20 patients that received treatment developed leukemia due to insertional mutagenesis (Hacein-Bey-Abina, von Kalle et al. 2003; Hacein-Bey-Abina, Von Kalle et al. 2003; Ott, Schmidt et al. 2006; Cavazzana-Calvo, Payen et al. 2010; Hacein-Bey-Abina, Hauer et al. 2010; Stein, Ott et al. 2010), despite preclinical trials predicting the risk of mutagenesis caused by the gammaretroviral vector to be extremely low (Kohn, Sadelain et al. 2003). Insertional mutagenesis can be caused either by read-through transcription of downstream genes resulting in increased transcripts of neighbouring genes, dysregulaton of neighbouring genes through the action of viral enhancers activating distant promoters or by the disruption of open-reading frames (Nienhuis, Dunbar et al. 2006; Bohne and Cathomen 2008).

In four out of five patients with X-linked SCID, T cell leukemia was caused by integration near the *LMO2* gene, which seems to be a hot spot for the

gammaretroviral vector (Hacein-Bey-Abina, Von Kalle et al. 2003; Hacein-Bey-Abina, Garrigue et al. 2008). Somewhat surprising, all five patients were X-linked SCID and none of the ADA-SCID patients have so far developed leukemia, despite the gene therapy protocols and the integration of the gammaretroviral vector being similar (Aiuti, Cassani et al. 2007; Deichmann, Hacein-Bey-Abina et al. 2007). It has been speculated that the reason for this is due to the X-linked disease background that might predispose for malignant transformation.

So far the lentiviral vector has not been shown to have the same preference of integration as the gammaretroviral vector (Biffi, Bartolomae et al. 2011). Even so, the random integration of retroviral vectors is a major safety concern in the clinical setting and gene transfer strategies with the aim of site-specific integration is under development and highly warranted, e.g. using zinc-finger nuclease (ZFN) (Lombardo, Genovese et al. 2007).

Osteopetrosis

Human osteopetrosis is a heterogenous group of genetic disorders characterized by abnormally dense bones due to a varying degree of dysfunctional osteoclasts and defective bone resorption (Van Wesenbeeck and Van Hul 2005). Osteopetrosis, also referred to as marble bone disease, occurs in several forms, where the most severe form is known as Infantile Malignant Osteopetrosis (IMO) (Askmyr, Flores et al. 2009; Stark and Savarirayan 2009).

IMO

IMO is a rare congenital disorder affecting approximately 1:300 000 children per year worldwide, except in Costa Rica where it is more common (3.4:100 000) (Fasth and Porras 1999; Balemans, Van Wesenbeeck et al. 2005). The lethal disorder is associated with an increased number of non-functional osteoclasts and the absence of bone resorption results in accumulation of sclerotic bone leading to abnormal bone marrow cavity formation insufficient to support hematopoiesis. Affected children suffer from bone marrow failure, anemia, thrombocytopenia, hepatosplenomegaly, immune dysfunction and recurrent infections (Wilson and Vellodi 2000). Furthermore, the impaired remodeling of the developing bone results in compression of cranial nerves which leads to neurological impairment, affecting especially vision and hearing which may progress to blindness and deafness (Steward 2003). Despite the increased bone mass the IMO children suffer from recurrent pathological fractures due to the abnormal remodeling of the primary woven bone to lamellar bone. They also have disturbed tooth eruption, a process dependent on osteoclast activity for creating a path in the jaw for the developing tooth (Helfrich 2005).

Molecular description of IMO

IMO is a monogenetic autosomal recessive disorder and can be caused by various mutations resulting in impaired resorption capacity of the osteoclast. The genes

known to be involved are *TCIRG1, CLCN7* and *OSTM1* but additional loci remain to be identified and characterized (Mazzolari, Forino et al. 2009).

The TCIRG1 gene

The most common gene mutated, affecting over 50% of the patients is the *TCIRG1* gene. The 7.9kb gene is located on chromosome 11q13.4-q13.5 and encodes two protein isoforms, OC116 and TIRC7, with different functions (Heinemann, Bulwin et al. 1999). The OC116 transcript codes for an a3-subunit of the V-ATPase; the multi-subunit proton pump involved in the transport of H+ to acidify the resorption area (Frattini, Orchard et al. 2000; Kornak, Schulz et al. 2000; Sobacchi, Frattini et al. 2001). The proton pump is composed of two domains (V_1 and V_0) that couple the energy from ATP hydrolysis (cytosolic V_1) to the translocation of protons from the cytoplasm to the resorption pit (transmembrane V_0) and can thereby pump the hydrogen ions against a high concentration gradient, creating the low pH required (Blair, Teitelbaum et al. 1989). The a3-subunit is primarily expressed in osteoclasts but also in the liver, kidney, brain, lung, spleen and muscle tissue. However, the highest expression level is in the osteoclasts, which may explain the osteopetrotic phenotype of the *TCIRG1* mutated patients (Manolson, Yu et al. 2003). The TIRC7 isoform is a T cell membrane protein involved in T cell activation (Utku, Heinemann et al. 1998; Utku, Boerner et al. 2004; Utku, Heinemann et al. 2006).

The CLCN7 gene

The CLCN7 gene encodes the chloride channel involved in the acidification process in conjunction with the vacuolar pump in the osteoclast. Mutations in the CLCN7 gene are less common, affecting around 15% of IMO patients, and loss of function results in the same severe phenotypes as seen with TCIRG1 deficiency (Kornak, Kasper et al. 2001; Frattini, Pangrazio et al. 2003). A milder form called intermediate autosomal recessive osteopetrosis also exists, in which some residual function of the chloride channel remains (Campos-Xavier, Saraiva et al. 2003).

The OSTM1 gene

Defects in the *OSTM1* (Osteopetrosis-associated transmembrane protein 1) gene also leads to the pathology of IMO. This mutation is seen only in 1-3% of the patients and they suffer from severe CNS involvement with cerebral atrophy in addition to the classical symptoms known from IMO (Pangrazio, Poliani et al. 2006). The exact function of the protein is not known but evidence suggests that it is required for the function of the chloride channel. Both *Clcn7* and *Ostm1* form a

complex in the ruffled border of the osteoclast, and Ostm1 might function as a β-subunit of Clcn7 necessary for protein stability (Lange, Wartosch et al. 2006).
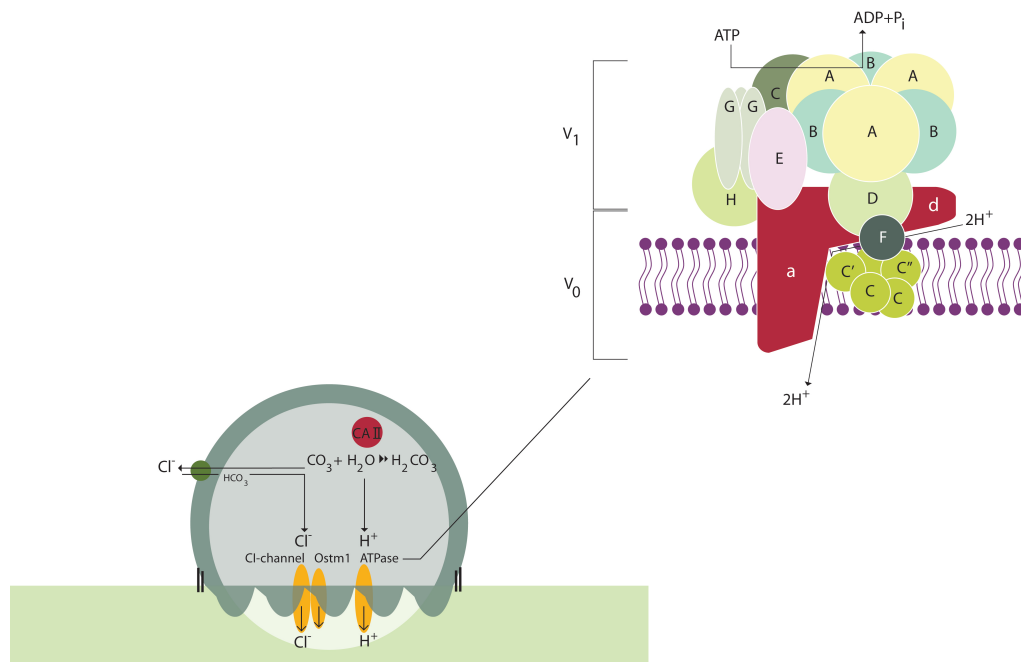
Figure 5. Mechanism of OC-mediated bone resorption

During resorption the activated OC undergoes cytoskeletal rearrangements to form a specialized sealing zone called the ruffled border that binds to the underlying bone and creates a closed compartment. The bone is degraded through acidification, a process mediated by a vacuolar proton pump that releases hydrogen ions creating a low pH in the compartment. This is followed by degradation of the matrix by cathepsin K and metalloproteinases. The figure also shows the V-ATPase consisting of the V_1 domain, formed by three A-, B-, and G-subunits and C, D, E, F and H subunits and the V_0 domain, formed by a, c, c`, c`` and d subunits.

The only curative treatment available today for IMO is HSCT (Coccia, Krivit et al. 1980; Solh, Da Cunha et al. 1995; Fasth and Porras 1999; Driessen, Gerritsen et al. 2003; Askmyr, Fasth et al. 2008).

The first evidence of the OC being of hematopoietic origin and OC disorders curable with BM transplantation was in the 1970s when osteopetrotic microphtalamic and grey-lethal mice were rescued by parabiosis to normal littermates or by transplantation of wild type (WT) BM and spleen cells (Walker 1973; Walker 1975; Walker 1975). Shortly after, the first HSCT was performed in osteopetrotic children and since then many patients have received cell therapy with varying success (Ballet, Griscelli et al. 1977; Coccia, Krivit et al. 1980; Driessen, Gerritsen et al. 2003). The long-term outcome of patients depends on the age at transplantation and type of donor used. The younger the child receiving the therapy is the better the outcome. In 2003, patients that received HLA-matched donors had almost an 80% 5-year disease-free survival but less than 40% when an alternative donor was used (Gerritsen, Vossen et al. 1994; Fasth and Porras 1999; Driessen, Gerritsen et al. 2003). However, results from the transplantations are improving and the probability of 5-year disease-free survival after transplantation using a matched unrelated donor (MUD) is today 80% and 65% when using a haplo-identical donor (Dr Ansgar Schulz, personal communication). However, with gene therapy the patient´s own stem cells could be used, eliminating the need of finding suitable donors. It has been shown that patients with IMO have a high number of $CD34^+$ cells circulating in the PB, which could be harvested and transduced without any need for prior mobilization (Steward, Blair et al. 2005).

If untreated, the children die during childhood from severe anemia, hemorrhage and infections due to bone marrow failure (Fasth and Porras 1999; Wilson and Vellodi 2000).

Mouse models used in this thesis

The mouse is a well-established experimental model frequently used in biomedical research. Humans and mice share a similar genome and physiology, making it possible to study diseases and complex physiological systems *in vivo* and thereafter extrapolate the findings to human biology. Especially for HSC research, mouse models have been invaluable tools since defining a true stem cell is based on its capacity of long-term self-renewal and repopulation of the complete hematopoietic system in the recipient. Furthermore, in HSC targeted gene therapy, the study of new vectors *in vivo* is made possible and long-term efficacy and safety can be assessed. The mouse genome can relatively easily be manipulated to induce diseases that do not naturally develop in mice. Presently thousands of inbred strains and genetically engineered mice exist, e.g. transgenic mice containing additional DNA to study gain of function and knock out (KO) models (conventional or conditional/inducible KO) to study loss of function (Nguyen and Xu 2008).

In this thesis three different mouse models have been used to study IMO in murine and human cells; the oc/oc and RANK KO mouse models, both having an osteopetrotic phenotype (containing non-functioning OCs or no OCs respectively) and the immunodeficient NSG mouse, lacking B- and T-lymphocytes and natural killer cells (Seifert and Marks 1985; Dougall, Glaccum et al. 1999; Shultz, Ishikawa et al. 2007).

The oc/oc mouse model

The oc/oc mouse has a spontaneous deletion in the *Tcirg1* gene located on chromosome 19 (Seifert and Marks 1985; Scimeca, Franchi et al. 2000). The mice exhibit the same symptoms as seen in humans, and develop a severe osteopetrotic phenotype due to the lack of osteoclastic V-ATPase activity and thus no resorptive function. They display an increased bone mass density and contain a high number of non-functional OCs compared to normal littermates (Marks, Seifert et al. 1984).

Impairment in hematopoiesis is also observed. A block in pro-B to pre-B cell development results in reduced numbers of mature B cells and the reason for this seems to be the altered microenvironment´s inability to produce IL-7, which can be partially corrected by exogenous IL-7 (Blin-Wakkach, Wakkach et al. 2004; Blin-Wakkach, Wakkach et al. 2006). The survival of the osteopetrotic mouse rarely exceeds three to four weeks, but as for the affected patients, long-term survival can be provided with HSCT (Johansson, Jansson et al. 2006). Also HSCT *in utero* has been shown to rescue oc/oc mice and reverse the osteopetrotic phenotype, in this case including preservation of vision, which underlines the importance of early treatment (Frattini, Blair et al. 2005). Research on the oc/oc mouse has proven that the osteopetrotic phenotype also can be reversed with gene therapy. Oc/oc fetal liver cells transduced with an oncoretroviral vector, containing the functional *Tcirg1* gene, were transplanted into one-day-old mice and 50% survived long term with an almost complete normalization of the skeletal phenotype (Johansson, de Vries et al. 2007).

The RANK KO mouse model

The RANK receptor is expressed on the surface of osteoclast precursors and after the binding of RANKL, in the presence of M-CSF, the progenitors differentiate and become active mature osteoclasts (Lacey, Timms et al. 1998; Yasuda, Shima et al. 1998). In the RANK KO mouse model, the RANK allele is deleted which results in the mouse being completely devoid of osteoclasts (Dougall, Glaccum et al. 1999). The mice suffer from impaired bone resorption giving them an osteopetrotic phenotype. A reduction in B lymphocytes in the spleen, and an impaired lymph node formation, are observed (Dougall, Glaccum et al. 1999). Transplanting the mice with WT BM cells or genetically corrected spleen cells, corrects the osteopetrotic phenotype (Li, Sarosi et al. 2000).

The NSG mouse model

The NSG (NOD/LtSz-scid IL-2R gamma null) mouse model is a further development of the previous model, the immunodeficient NOD-SCID mouse. Besides the SCID (Prkdc^{scid}) mutation and NOD strain background, there is a null-mutation at the interleukin-2 receptor (IL-2R) γ-chain locus, which makes it the most immunodeficient mouse model present today (Cao, Shores et al. 1995; Shultz, Schweitzer et al. 1995; Shultz, Ishikawa et al. 2007). The IL-2R is

essential for signal transduction after the binding of the cytokine IL-2 and the dysfunctional IL-2R results in impaired T- and B-lymphocytes and lack of natural killer cells (Cao, Shores et al. 1995; DiSanto, Muller et al. 1995; Ohbo, Suda et al. 1996; Ishikawa, Yasukawa et al. 2005). Furthermore, the IL-2R γ-chain is also an essential component in multiple cytokine receptor complexes for IL-4, IL-7, IL-9, IL-15 and IL-21, resulting in additional defects in the innate immune system (Shultz, Schweitzer et al. 1995). The lack of immune system and the long lifespan of the mice (> 90 weeks) enable the study of human HSCs in vivo long-term (Ito, Hiramatsu et al. 2002; Ishikawa, Yasukawa et al. 2005; Shultz, Lyons et al. 2005). This is a great improvement compared to the previous NOD-SCID model, which had a short lifespan and residual NK cell activity that compromised the level of engraftment (Shultz, Schweitzer et al. 1995). There are several IL-2R $\gamma^{-/-}$ mouse strains and the strain background affects the engraftment and function of human cells in the mice.

Limitations with the NSG mice include the lack of cross-reactive cytokines between human and mouse cells. This results in a decreased production of myeloid cells and B-cells. New immune-compromised mouse models have been developed, where the human genes have been added, coding for the species-specific cytokines, e.g. SCF, IL-3, GM-CSF (Billerbeck, Barry et al. 2011) and M-CSF (Rathinam, Poueymirou et al. 2011).

Aims of the present investigation

Paper I

To investigate whether conditioning with radiation prior to neonatal HSCT can be replaced by busulfan in the oc/oc mouse model and if vision then can be preserved.

Paper II

To investigate if neonatal HSCT without any prior conditioning can reverse osteopetrosis in the oc/oc mouse model and to study if there is any selective recruitment of donor cells to the osteoclast lineage.

Paper III

To restore OC function by lentiviral gene transfer of *TCIRG1* into PB CD34$^+$ cells from IMO patients.

Paper IV

To study if functional OCs are required for the maintenance and function of HSCs in the adult mouse.

Summary of results

Paper I

In this study we wanted to develop a clinically relevant model for cell therapy in the oc/oc mouse. Previous transplantation studies in oc/oc mice have used total body irradiation (TBI) to eliminate endogenous hematopoietic cells prior to transplantation. However, this is not a clinically performed conditioning regimen for transplantation of patients with IMO due to side effects. Most IMO patients are preconditioned with busulfan and cyclophosphamide, often with the addition of other cytotoxic drugs, before HSCT. We treated pregnant mice at day 18.5 with two concentrations of busulfan (15 and 7,5 mg/kg) and transplanted their litters with 1 million lineage-depleted BM cells intravenously (iv) or intraperitoneally (ip). Busulfan at 15 mg/kg was toxic to oc/oc mice but almost all oc/oc mice conditioned with busulfan at 7.5 mg/kg survived past the normal lifespan with 10% engraftment, correction of the skeletal phenotype, and normalization of PB lineages. Furthermore, because irradiation of neonatal mice causes retinal degeneration, we wanted to investigate whether conditioning with busulfan prior to HSCT might prevent this. We found that busulfan did not have adverse effects on the vision and retina as determined by the visual tracking drum test and eye histopathology. In conclusion, these findings show that low-dose busulfan conditioning and neonatal HSCT lead to prolonged survival of oc/oc mice, reverse osteopetrosis and prevent blindness even at low engraftment levels.

Paper II

Nonablative Neonatal Bone Marrow Transplantation Rapidly Reverses Severe Murine Osteopetrosis Despite Low-Level Engraftment and Lack of Selective Expansion of the Osteoclastic Lineage.

Cytoreductive conditioning is in most cases required before transplantation to create space for transplanted cells in the hematopoietic niches in the BM but there are many side effects involved. In this study we aimed to determine if neonatal transplantation without any prior conditioning could reverse osteopetrosis and rescue oc/oc mice, not only to further advance the development of cell and gene therapy for IMO, but also to gain more insight into the role and capacity of osteoclasts in remodeling of bone tissue *in vivo*. 1-day old unconditioned oc/oc mice were transplanted with normal nonmanipulated (5 million) or lineage-depleted (1 or 5 million) BM cells. We found that more than 85% of oc/oc mice transplanted with 5 million lineage-depleted cells survived long term (up to a year) with an engraftment of 3-5% in PB, whereas almost all oc/oc mice in the other groups, including untreated mice, succumbed after 3 to 5 weeks. At 3 w engraftment in the BM was 1-2% but the cellularity had increased 60-fold compared to non-treated oc/oc and RANKL and M-CSF expression in the BM was normalized. Histopathology and micro-CT revealed almost complete reversal of osteopetrosis after 4 weeks. *In vitro* studies showed that bone resorption by osteoclasts from transplanted oc/oc mice was 14% of transplanted controls and immunofluorescence microscopy revealed that resorption was mainly associated with osteoclasts of donor origin. Lineage analysis of BM, PB and spleen did not provide any evidence for selective recruitment of cells to the osteoclastic lineage. The vision was also preserved in transplanted oc/oc mice as determined by a visual tracking drum test. These findings show that engraftment of only a small fraction of normal cells is sufficient to tip the *in vivo* balance between bone formation and bone resorption in this severe model of osteopetrosis, which is of great importance for the development of HSC-targeted gene therapy for IMO.

Paper III

TCIRG1 Lentiviral Gene Transfer to Peripheral Blood CD34$^+$cells restores Osteoclast Function in Infantile Malignant Osteopetrosis.

Molecular Therapy, manuscript under revision.

The aim of this study was to restore the resorptive function of IMO OCs by lentiviral mediated gene transfer of the *TCIRG1* cDNA. CD34$^+$ cells from PB of five IMO patients and from normal cord blood (CB) were transduced with lentiviral vectors expressing *TCIRG1* and GFP under a SFFV promoter. Transduced cells were expanded in culture and subsequently differentiated on bone slices to mature GFP$^+$ OCs. Here we show that vector-corrected IMO osteoclasts express mRNA and protein levels of TCIRG1 comparable to controls. The IMO-rescued OCs were able to generate increased Ca^{2+} release and bone degradation products such as CTX-1 into the media and showed clearly visible resorption pits, in contrast to IMO controls. When investigating vector-mediated TCIRG1 expression during osteoclastogenesis, we found that TCIRG1 expression was first observed when the mature OCs were formed (day 7), in contrast to GFP that was expressed from the start. This indicates a possible post-transcriptional regulation of TCIRG1 during osteoclast differentiation. Furthermore, transduced CD34$^+$ cells from CB were transplanted into sub-lethally irradiated NSG mice and analyzed 6-10 weeks post-transplantation. We found efficient long-term human reconstitution in PB and BM. No difference in lineage distribution of BM cells was observed, indicating that the vector expressing TCIRG1 does not skew the differentiation potential. This paper provides the first proof-of-principle of lentiviral-mediated correction for IMO.

Paper IV

Osteoclast Mediated Bone Resorption is not Essential for Hematopoietic Stem Cell Maintenance in Adult Mice.

Manuscript in preparation.

The HSC niche is a complex environment involving many different regulators, one key regulator being the OB. Besides the tightly coupled relationship between the OB and the OC, the direct role of the OC in the regulation of the niche in the adult mouse after its formation, is more controversial. To shed more light on this process we transplanted HSCs from two osteopetrotic mouse models, with lack of osteoclasts (RANK KO) or defective osteoclast function (oc/oc), to normal adult mice and examined the bone phenotype and hematopoiesis in the recipients. After transplantation a donor chimerism of 97-98% was obtained and 15 weeks post transplantation both adult mouse models developed a mild osteopetrotic phenotype. The oc/oc recipient had increased numbers of OCs *in vitro* and *in vivo* and the RANK KO recipient had no OCs *in vitro* and reduced numbers *in vivo*. The bone resorption capacity was equally reduced in both groups and was determined by measuring marker CTX-I. Further, micro-CT revealed a significant increase in bone volume and bone strength of femur and neck in both mouse models indicating that bone formation had been uncoupled from bone resorption after 15 weeks. This is important because we were interested in studying the direct effect of the OC on hematopoiesis and not the effect through the OB. We found no change in the number of BM cells between the groups. The number of progenitors measured by CFUs per femur were reduced in RANK KO recipients but unchanged in oc/oc recipients compared to controls. When HSCs were immunophenotypically analyzed we found a reduction in HSC numbers in oc/oc and RANK KO recipients compared to controls, but when the same cells were functionally analyzed by competitive secondary transplantation they performed equally well as controls. Our results indicate that OC function is not essential for HSC maintenance in adult mice.

General discussion

Therapeutic effect despite low level engraftment – lack of selective advantage

Most often myeloablation is required prior to HSC targeted gene therapy to reduce the number of endogenous HSCs competing for space in the stem cell niches (Aiuti, Slavin et al. 2002; Gaspar, Bjorkegren et al. 2006; Ott, Seger et al. 2007). However, in some cases the normal or gene-corrected cells have an advantage over the mutated cells eliminating the need for any myeloablation. In X-linked SCID there is a strong selective expansion of the corrected T cells, resulting in a remarkable therapeutic effect despite the low level of stem cell engraftment obtained in the absence of conditioning (Cavazzana-Calvo, Hacein-Bey et al. 2000; Schmidt, Hacein-Bey-Abina et al. 2005). Furthermore, in the ADA-deficient mouse model, nonablative transplantation revealed that despite absence of selective expansion of T cells, a unique trans-rescue occurred by the normal cells, that managed to produce enough ADA enzyme to rescue the recipient ADA-deficient lymphocytes (Carbonaro, Jin et al. 2008). However as previously mentioned, the clinical efficacy has been limited due to low engraftment levels hence ADA-SCID patients receive nonmyeloablative conditioning prior to SCT (Aiuti, Slavin et al. 2002).

In paper two we wanted to determine if there was any evidence of selective expansion of the osteoclast lineage in the oc/oc mouse model after nonablative SCT. However, when comparing the frequency of donor-derived cells there was no difference between oc/oc and control mice in the pre-OC CD11b$^+$/Gr^{low} cell population in PB, spleen and BM, indicating no such selective recruitment (Flores, de Vries et al. 2010). We hypothesize that the reason behind the rapid reversal of osteopetrosis could be due to the nature of the OC formation, that one normal monocyte may fuse with a number of disease affected cells and rescue the entire osteoclast function (see figure 6). It has been suggested that the number of nuclei correlates with the resorption capacity but the exact mechanisms behind this remain unknown (Piper, Boyde et al. 1992; Boissy, Saltel et al. 2002).
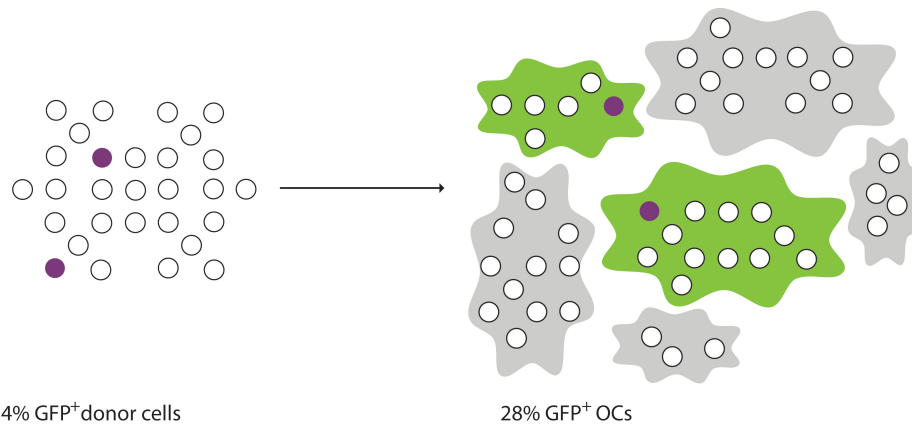
4% GFP⁺donor cells

28% GFP⁺ OCs

Figure 6. Hypothesis: Rescue of the OC function despite low level of engraftment
In paper two we observed 28% GFP⁺ OCs despite only 4% engraftment of GFP⁺donor cells after nonablative HSCT. If we first assume, that GFP⁺ and GFP⁻ pre-OCs fuse randomly, second that the average number of nuclei per OC formed is 8, and third, that the OCs formed become GFP⁺ even if only one of the fusing cells is GFP⁺, the probability that the OCs formed are GFP⁻ is: $(1-0.04)^8 = 72\%$; thus the remaining 28% of the multi-nucleated cells should be GFP⁺, a calculation which is consistent with our observations. White cells: GFP⁻ recipient cells, purple cells: GFP⁺ donor cells. Grey cells: nonresorptive OCs. Green cells: resorptive OCs.

Gene transfer efficiency and functional correction

The results from the oc/oc mouse model, showing that only a small amount of normal cells were sufficient to correct the severe phenotype, are very important and encouraging when aiming towards gene therapy for IMO patients (Askmyr, Holmberg et al. 2009; Flores, de Vries et al. 2010). We investigated if the rescue of nuclei in the OC is true also for human cells by culturing CD34⁺ IMO cells with different ratios of CB CD34⁺ cells to determine the OC function *in vitro* and our preliminary results show that 5% CB CD34⁺ cells is sufficient to largely restore the resorption capacity of OCs generated from these cultures (unpublished data). Furthermore, we will culture CD34⁺ IMO cells with different ratios of gene-corrected CD34⁺ IMO cells to determine the correlation between gene transfer efficiency and functional correction *in vitro*.

Regulation of TCIRG1 gene expression during osteoclastogenesis

For the pre-OCs to differentiate and form mature OCs, two hematopoietic cytokines are essential; M-CSF and RANKL. RANKL binds to the RANK receptor on pre-OCs and induces signaling pathways that lead to the upregulation of certain osteoclast specific genes, e.g. *Tcirg1* gene (Blair, Robinson et al. 2005).

In pre-OCs, the *Tcirg1* gene is repressed by a poly (ADP-ribose) polymerase-1 (PARP-1) (Beranger, Momier et al. 2006), but upon RANKL treatment, the PARP-1 protein is degraded and *Tcirg1* gene expression is upregulated by junD proto-oncogene (junD) and Fos-related antigen (Fra-2) (Beranger, Momier et al. 2007). The transcriptional regulation of the *Tcirg1* gene has been well studied but so far no post-transcriptional regulation has been described.

When a transgene is driven by a strong viral promoter, e.g. the SFFV promoter it is often expressed ectopically at differentiation stages where expression is normally not observed. In manuscript three, we were therefore expecting the TCIRG1 protein to be expressed in the pre-OCs as well as in mature OCs. However, during osteoclastogenesis of gene-corrected IMO cells we observed that the TCIRG1 protein was expressed in the same way as the endogenous protein is in normal OCs. The protein became visible on the western blot first on day 7, at the same time as mature OCs formed, whereas the co-expressed GFP was present at all times during OC differentiation. When the cells were cultured without RANKL no expression of TCIRG1 was observed (unpublished data). These observations suggest that the TCIRG1 is regulated at a post-transcriptional level, and that this is dependent on RANKL. It is possible that the subunit-proteins first become expressed during the assembly of the vacuolar proton pump, or that the TCIRG1 is degraded in pre-OCs up until the mature OCs are formed.

If vector mediated TCIRG1 expression is regulated in the same manner as the endogenous protein, the TCIRG1 would only be expressed in mature OCs, which would eliminate the need for tissue-specific vectors in the clinical setting.

The OCs in adult hematopoiesis

Hematopoietic and bone tissues are anatomically and functionally correlated. Alterations in one system cause impairment in the other, e.g. in osteopetrosis where dysfunctional OCs cause impairment in hematopoiesis.
Osteopetrosis can be divided into osteoclast-rich, with a high number of non-functional OCs and osteoclast-poor forms, lacking OCs. Both types result in hematopoietic impairment e.g. anemia and pancytopenia (Del Fattore, Cappariello et al. 2008). However, in osteoclast-rich osteopetrosis the space for hematopoietic tissue is even more limited due to abnormal fibrous tissue filling up the small spaces left in the osteopetrotic bone making the osteopetrotic phenotype even

more severe (Taranta, Migliaccio et al. 2003; Del Fattore, Peruzzi et al. 2006; Del Fattore, Capannolo et al. 2010). This is not seen in osteoclast-poor osteopetrosis, resulting in a better BM quality (Sobacchi, Frattini et al. 2007). The exact mechanisms underlying these processes remain to be illuminated but this could explain why the oc/oc mice have a shorter lifespan than the RANK KO mice.

The significance of the OCs in the niche regulation is under investigation but so far the recent major findings are controversial and remain to be clarified. Recently, a publication showed that when OC function was inhibited with the bisphosphonate alendronate (ALN), the HSC number was reduced, the authors concluding that OC function is fundamental in the HSC niche (Lymperi, Ersek et al. 2011). In manuscript four, we wanted to shed some light on how a reduction in OCs or the abolishment of OC resorptive function affect HSCs and the maintenance of hematopoiesis in the BM of adult mice. We investigated this by avoiding the use of pharmaceutical agents and instead utilized two genetically modified mouse models to modulate OCs in adult mice. WT mice were lethally irradiated and subsequently transplanted with FL cells from two osteopetrotic mouse models, the oc/oc and the RANK KO model. A donor chimerism of 97-98% was obtained and mild osteopetrosis developed in both recipients, however it cannot be ruled out that the remaining endogenous WT BM cells may have mitigated the phenotype developments in these new adult mouse models of osteopetrosis. Four months post-transplantation, we analyzed whole BM from both oc/oc and RANK KO recipients by FACS, and observed a slight but significant reduction in the amount of LSK SLAM cells. However, when the HSCs were transplanted in a competitive setting with WT BM cells, there was no impaired function compared to controls, thus our conclusion is that the OCs appear not to be essential for stem cell maintenance and function in the adult mouse.

In theory, the optimal mouse models to investigate how OC-mediated resorption or absence of OCs affect the adult hematopoiesis, would be to generate inducible KO models, targeting the *Tcirg1* gene and the *Rank* gene respectively.

Future perspectives

The ultimate goal of this project is to provide IMO patients lacking suitable donors an alternative treatment to SCT for this otherwise severe disease with fatal outcome. The results obtained in the oc/oc mouse show that engraftment of only 5-10% of normal or gene-modified cells is sufficient to correct IMO (Johansson, de Vries et al. 2007; Askmyr, Holmberg et al. 2009; Flores, de Vries et al. 2010). After demonstrating correction of IMO in patient cells by HSC targeted gene therapy as a proof of concept, we are now aiming towards a clinical trial.

Xenograft transplantations

Currently ongoing experiments aim to prove that the PB CD34$^+$ cells from IMO patients are true HSCs by transplanting them into NSG mice and investigate long-term engraftment potential in PB and BM. CD34$^+$ cells from IMO patients and from normal CB will be transduced with the therapeutic lentiviral vector and transplanted into NSG mice, to make sure that HSC gene transfer has occurred and that the ectopic expression of TCIRG1 does not interrupt normal hematopoiesis *in vivo*.

Preclinical research will also involve optimization of the mouse model used in xenotransplantation. Although the NSG mouse has provided us with information about efficacy and vector toxicity *in vivo*, the limitations regarding myeloid differentiation remain. We are currently investigating if human OCs can be formed *in vivo* in the NSG mouse, as this has not previously been shown. NSG mice are transplanted with transduced CD34$^+$ cells and analyzed for TRAP$^+$, GFP$^+$ OCs in bone sections. If the development of OCs is poor in the NSG model due to the species-specific murine M-CSF, we will replace the currently used mouse model with the humanized CSF-1 mouse (Rathinam, Poueymirou et al. 2011), in which the human M-CSF gene has been inserted, resulting in production of human M-CSF and increased levels of human monocytes and macrophages after transplantation of CD34$^+$ cells. Furthermore, we are in the process of developing a

novel osteopetrotic immune deficient mouse model by crossing the oc/oc mouse strain with the NSG (or humanized CSF-1 mice). If this project is successful it may give rise to an ideal animal model to study disease-correcting strategies in IMO patient cells *in vivo*.

Development of clinical vectors

Taking safety into consideration, the clinical vectors will be third-generation lentiviral vector pseudotyped with VSV-G and contain a cellular promoter derived from human genes, e.g. phosphoglycerate kinase (PGK) or elongation factor 1-alpha (EF1-α) promoter. By replacing the viral SFFV promoter, the risk of activating neighbouring genes is significantly reduced (Zychlinski, Schambach et al. 2008). The clinical vectors where the *TCIRG1* is driven by PGK or EF1-α will be functionally tested to determine the expression level needed for the same degree of functional correction as we have observed with the SFFV promoter. Tissue specific-promoters controlling gene expression in a tissue-dependent manner could be of interest if it turns out that the *TCIRG1* expression is not regulated at a post-transcriptional level (discussed above). The vectors will also be analyzed for integration sites and clonal proliferation by transplanting NSG mice with gene-corrected cells and follow them long-term and subsequently performing linear amplification–mediated (LAM)-PCR.

Patient selection and clinical procedure

The clinical gene therapy trial will be offered to children diagnosed with IMO regardless of age, that lack a suitable stem cell donor e.g. sibling or MUD. Experienced pediatricians will be able to choose between haplo-identical BMT or gene therapy and evaluate the optimal treatment option for each individual child.
As it has been shown that children with IMO have a higher number of PB CD34^{+} cells compared to normal conditions, the CD34^{+} will be harvested by exchange transfusion without prior mobilization regimen (Steward, Blair et al. 2005).
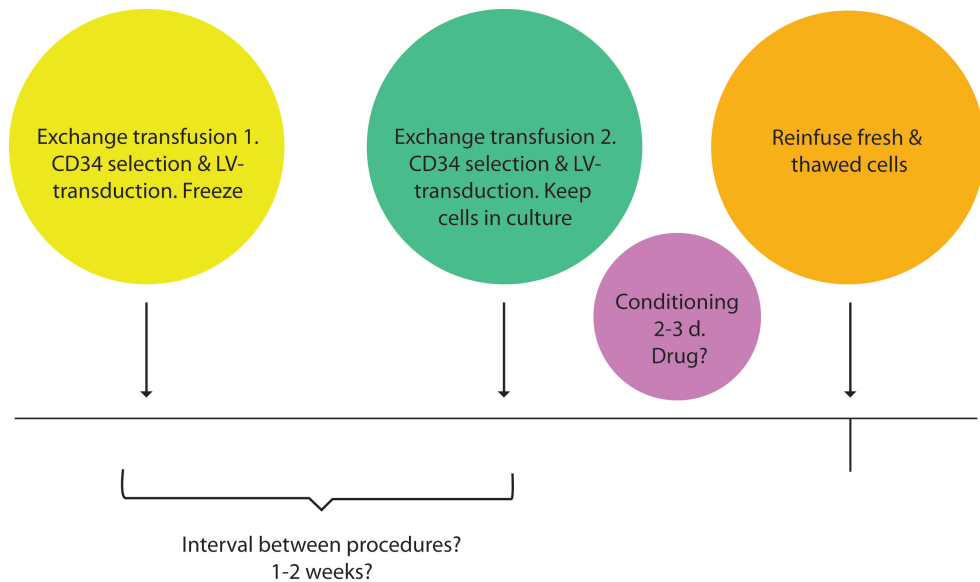
Figure 7. Proposed outline of clinical gene therapy procedure

To ensure that we obtain enough PB CD34^{+} cells, exchange transfusion will be performed two times. This means that PB CD34^{+} cells will be harvested and transduced with the optimal therapeutic lentiviral vector chosen from the preclinical experiments. Transduced CD34^{+} cells from the first exchange transfusion will temporarily be frozen. Approximately 2 weeks later, the second round of exchange transfusion will be performed and depending on the time period of the conditioning regimen, the cells will be kept in culture or frozen before being re-infused into the patient. It is still unclear whether myeloablation is needed for stable engraftment, but to ensure sufficient space for the gene-corrected cells in the niche, low-dose busulfan will be administered, as used in the oc/oc model and in the ADA-SCID and CGD trials (Aiuti, Slavin et al. 2002; Ott, Schmidt et al. 2006; Askmyr, Holmberg et al. 2009). After the gene therapy treatment, the patients will regularly be analyzed for the clonal composition of hematopoiesis (Schmidt, Schwarzwaelder et al. 2007).

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Populärvetenskaplig sammanfattning på svenska

Min avhandling har fokuserat på att öka förståelsen för den ovanliga, allvarliga genetiska sjukdomen infantil malign osteopetros (IMO), med målet att utveckla genterapi som en ny behandlingsform för sjukdomen. IMO finns redan vid födseln och orsakas av att osteoklasterna, cellerna ansvariga för nedbrytning av ben, inte fungerar. Sjukdomen kan orsakas av flera olika gendefekter men i över hälften av fallen är det en gen som kallas *TCIRG1* som är muterad. Icke-fungerande osteoklaster leder till en kraftigt ökad bentäthet som påverkar blodkärl och nerver som komprimeras på grund av bristande hålutrymmen i skelettet. Trots bildandet av mer ben är benstrukturen skör och benbrott är vanliga. Kompression av bland annat syn- och hörselnerv leder till blindhet och dövhet. Slutligen upphör benmärgen att fungera och barn utan behandling dör vid ca fem års ålder, på grund av blodbrist och infektioner. Eftersom osteoklasterna har sitt ursprung i blodstamceller precis som övriga blodceller, kan sjukdomen botas genom benmärgstransplantation. På så sätt ersätts de defekta blodstamcellerna med stamceller från en frisk donator. Denna behandling är dock riskfylld och kräver att det finns passande donatorer tillgängliga.

I de första två arbetena har vi arbetat med en musmodell som också har en mutation i *Tcirg1* genen och uppvisar liknande symptom som finns i människa. Dessa möss lever bara fyra veckor om de inte transplanteras med friska blodstamceller. Både i människa och mus måste oftast stamcellsmottagaren förbehandlas i syfte att skapa utrymme för transplanterade stamceller. I **arbete ett** optimerade vi transplantationsproceduren så att den blev mer kliniskt relevant för fortsatt forskning. Strålning byttes ut mot låga doser cellgift, och stamceller administrerades intravenöst, vilket resulterade i att biverkningar av strålningen eliminerades och administrationssättet effektiviserades. I **arbete två** transplanterade vi stamceller utan att först förbehandla mössen och upptäckte att även då kunde vi uppnå tillräckligt benmärgsanslag för att rädda mössen och få en komplett normalisering av benmärg och skelett. Detta är ett viktigt fynd som underbygger valet av IMO som en lämplig sjukdomskandidat för utveckling av genterapi.

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Blodstamcellen har förmågan till självförnyelse och produktion av alla typer av celler i blodet under ett helt liv. Genom att korrigera dessa celler kan man återställa funktionen av alla osteoklaster som bildas. Grundidén med genterapi är att tillföra en normal genkopia till en cell som saknar eller har en defekt gen, så att patienten själv kan bilda ett funktionellt protein. Man har redan idag framgångsrikt behandlat vissa sällsynta sjukdomar som drabbar immunförsvaret med genterapi och har genom detta lyckats rädda patienter som annars inte skulle överlevt.

I **arbete tre** gick vi över till patientceller och använde oss av ett modifierat HIV virus som verktyg för att införa en normalt fungerande genkopia av *TCIRG1* till blodbildande humana stamceller. Här visar vi att funktionen hos de defekta osteoklasterna korrigerades med ca 70-80% och nedbrytningen av ben visas genom att mäta nivåer av bland annat kalcium i blodet.

I **arbete fyra** var vi intresserade av att belysa osteoklasternas roll och betydelse för blodstamcellernas reglering i benmärgen hos vuxna möss. För att undersöka detta använde vi oss av två musmodeller: en med icke-fungerande osteoklaster och en som helt saknar osteoklaster. Blodstamceller från dessa musmodeller transplanterades sedan in i friska vuxna möss och efter flera månader analyserade vi blodet. Våra resultat visade ingen funktionell skillnad mellan blodstamcellerna från osteoklastdefekta musmodeller och friska blodstamceller, vilket kan bero på att osteoklasterna inte har så stor betydelse för stamcellsfunktionen i vuxna möss.

Sammanfattningsvis är dessa studier viktiga för en ökad förståelse av sjukdomen IMO och genterapi som behandlingform. Vi har även fått mer insikt i osteoklasternas roll i regleringen av blodstamceller. Med mål att bota barn med IMO inom den nära framtiden, väntar först ytterligare försök för att testa nya säkrare vektorer i optimerade musmodeller som är bättre anpassade för att analysera patientceller *in vivo*.

Papers not included in this thesis

1. Askmyr M, **Flores C**, Fasth A, Richter J. Prospects for gene therapy of osteopetrosis. Current Gene Therapy, 2009, 9(3): 150-9.

2. Henriksen K, **Flores C**, Thomsen JS, Brûel AM, Thudium CS, Neutzsky-Wulff AV, Langenbach GEJ, Sims N, Askmyr M, Martin TJ, Everts V, Karsdal MA, Richter J. Dissociation of Bone Resorption and Bone Formation in Adult Mice with a Non-Functional V-ATPase in Osteoclasts Leads to Increased Bone Strength. PLoS One. 201, 6(11): 27482.

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