Development of Novel Models for Studying Osteoclasts

Christian Schneider Thudium

LUND UNIVERSITY

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Umeå, Sweden
Abstract

This thesis focuses on developing and characterizing novel models for studying osteoclasts with an emphasis on how mutations abolishing osteoclastic acidification affect osteoclast signaling and bone remodeling, as well as how to treat patients bearing these rare mutations.

Bone remodeling is under normal circumstances a tightly balanced process where resorption of bone by osteoclasts is followed by adequate amounts of bone formation by osteoblasts. Mutations in the TCIRG1 gene lead to severe autosomal recessive osteopetrosis (ARO) in both mice and man, characterized by lack of bone resorption as a result of abolished acid secretion, an increased number of non-resorbing osteoclasts, but normal or even increased bone formation.

The first two papers are based on studies in adult mice transplanted with stem cells from either osteoclast-rich osteopetrotic mice (oc/oc) or osteoclast-poor osteopetrotic mice (RANK KO). The first paper focuses on characterizing the bone and cellular phenotype in the adult osteopetrotic mouse model and find that bones, are both bigger and, in contrast to the endogenous oc/oc mouse model, stronger in mice transplanted with osteopetrotic stem cells compared to wild type transplanted mice. In the second paper we compare the osteoclast-rich osteopetrotic oc/oc transplanted model to adult mice transplanted with stem cells from the osteoclast-poor RANK KO mouse, and find that maintaining non-resorbing osteoclasts in vivo increases bone formation. In the third paper we investigate the role of bone matrix, cell stage and resorptive function on osteoclast mediated anabolic signaling. In paper IV human stem cells from patients with ARO were investigated and we provide the first proof-of-principle for lentiviral mediated correction of resorptive function in CD34+ derived osteoclasts from ARO patients.

The results can be devided into two exciting branges. Data obtained in the adult osteopetrotic mouse models encourage further development of molecules targeting the acidification process in osteoclasts when treating low bone mineral density diseases. Furthermore, the correctional results in the ARO patient cells are very promising, and will serve as basis for further development of clinical gene therapy of osteopetrosis.

Key words Osteoclast, Bone remodeling, Gene therapy, hematopoietic stem cells, Osteopetrosis, Osteoporosis
Development of Novel Models for Studying Osteoclasts

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Ulf Lerner, DDS., Ph.D.
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### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ADA</td>
<td>adenosine-deaminase</td>
</tr>
<tr>
<td>ADO</td>
<td>autosomal dominant osteopetrosis</td>
</tr>
<tr>
<td>AE2</td>
<td>anion exchanger II</td>
</tr>
<tr>
<td>ARO</td>
<td>autosomal recessive osteopetrosis</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMU</td>
<td>basic multicellular unit</td>
</tr>
<tr>
<td>BRC</td>
<td>bone remodeling compartment</td>
</tr>
<tr>
<td>CAII</td>
<td>carbonic anhydrase II</td>
</tr>
<tr>
<td>CGD</td>
<td>chronic granulomatous disease</td>
</tr>
<tr>
<td>CIC-7</td>
<td>chloride channel 7</td>
</tr>
<tr>
<td>CM</td>
<td>conditioned media</td>
</tr>
<tr>
<td>DC-STAMP</td>
<td>dendritic cell-specific transmembrane protein</td>
</tr>
<tr>
<td>FLC</td>
<td>fetal liver cell</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>IMO</td>
<td>infantile malignant osteopetrosis</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>MAR</td>
<td>mineral apposition rate</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>RANK</td>
<td>receptor activator of nuclear factor κB</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor κB ligand</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SFFV</td>
<td>spleen focus forming virus</td>
</tr>
<tr>
<td>SIN</td>
<td>self-inactivating</td>
</tr>
<tr>
<td>TCIRG1</td>
<td>T cell immune regulator 1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TRAP</td>
<td>tartrate resistant acid phosphate</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
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</table>
Articles Included in this Thesis

I) Dissociation of bone resorption and bone formation in adult mice with a non-functional V-ATPase in osteoclasts leads to increased bone strength

Kim Henriksen, Carmen Flores, Jesper S. Thomsen, Annemarie Brüel, Christian S. Thudium, Anita V. Neutzsky-Wulff, Gerling E. J. Langenbach, Natalie Sims, Maria Askmyr, Thomas J. Martin, Vincent Everts, Morten A. Karsdal and Johan Richter

_Plos One, 2011; 6(11): e27482_

II). A comparison of osteoclast-rich and osteoclast-poor osteopetrosis in adult mice sheds light on the role of the osteoclast in coupling bone resorption and bone formation.

Christian S. Thudium, Ilana Moscatelli, Carmen Flores, Jesper S. Thomsen, Annemarie Brüel, Natasja S. Gudmann, Ellen-Margrethe Hauge, Morten A. Karsdal, Johan Richter and Kim Henriksen


III) A specific subtype of osteoclasts secretes factors inducing nodule formation by osteoblasts.


_Bone, 2012; 51(3):353-61_

IV) Lentiviral gene transfer of TCIRG1 into peripheral blood CD34(+) cells restores osteoclast function in infantile malignant osteopetrosis.

Ilana Moscatelli*, Christian S. Thudium*, Carmen Flores, Ansgar Schulz, Maria Askmyr, Natasja Stæhr Gudmann, Nanna Merete Andersen, Oscar Porras, Morten Asser Karsdal, Anna Villa, Anders Fasth, Kim Henriksen and Johan Richter

_Bone, 2013; 57(1):1-9._

* These authors contributed equally to this work
Introduction

The osteoclast is an extraordinary cell type with the unique ability to degrade the calcified bone matrix. The non-redundant nature of the osteoclast often makes cell functionality the center of pathological disease states, such as osteoporosis and osteopetrosis, as well as an interesting target for therapeutic development.

In contrast to the layman perception of the skeleton as rather inert, bone is a living organ which is constantly degraded and rebuilt throughout life to maintain mineral homeostasis and repair microdamage hereby ensuring skeletal integrety; a process known as bone remodeling. This process involves the resorption of old bone by osteoclasts, and the formation of new bone by the osteoblasts. Bone remodeling is strictly regulated and characterized by a tight coupling between resorption and formation so that under normal circumstances resorbed bone is always replenished with equal amounts of new bone in the healthy adult. Hormonal changes or genetic defects can skew the balance and lead to pathological changes in bone. Mutations which affect osteoclastic acidification, result in defective bone resorption and leads to the severe hereditary disorder autosomal recessive osteopetrosis (ARO). These individuals have a high number of non-resorbing osteoclasts, and despite lacking bone resorption, have normal or increased bone formation, suggesting that the osteoclast, but not necessarily bone resorption is important for ongoing bone formation.

The thesis focuses on developing in vivo and in vitro osteoclast models for investigating how genetic defects in osteoclasts affect its regulation of bone remodeling, as well how to rescue osteoclast function in ARO by gene therapy mediated correction of mutational defects.
Skeletal Function

Bone, together with cartilage, makes up the human skeleton, and performs several important functions in the human body. The skeleton itself acts as structural support for the rest of the body and as attachment site for muscles and ligaments for locomotion. It serves as a protective scaffold to shield vital organs, including the bone marrow (BM). The skeleton also has a general metabolic function as a major source of inorganic ions, actively participating in maintenance of mineral homeostasis and acid-base balance. Moreover, the bones serve as a large reservoir of growth factors and cytokines. Finally, the bones provide the environment for hematopoiesis within the marrow cavities (Clarke, 2008)

In general the bones of the skeleton can be divided into two categories based on anatomy: flat bones, such as the skull and the mandible (which will not be discussed further), and long bones such as the femur, the tibia and the vertebrae (Clarke, 2008). A schematic view of a long bone is given in Figure 1. Long bones are made up of two different types of bone, namely cortical bone and trabecular bone (also known as cancellous bone). (Clarke, 2008). Approximately 80% of the human skeleton consists of cortical bone while 20% consists of trabecular bone; however, the ratio between the two types varies greatly between skeletal sites (Seeman and Delmas, 2006). The most prominent role of cortical bone is to ensure the mechanical properties of the skeleton, while trabecular bone, in addition to its mechanic role, also serves various metabolic functions (Seeman and Delmas, 2006). The varying roles of the two types of bone tissue also naturally influence on their respective remodeling rates.
Figure 1. Schematic drawing of a long bone
A schematic view of the structure and composition of a long bone. The bone is divided into epiphysis, metaphysis, and diaphysis. The bone consists of two kinds of bone, trabecular (cancellous), and cortical bone. The inner and outer parts of the bone are called the endosteum and the periosteum, respectively. The bone marrow is located in the cavity at the center of the bone. Adapted from (www.bbc.co.uk, 2014).
Bone Remodeling and Coupling

Frost described in what he termed intermediary organization separate distinct functions of bone cells in the skeleton such as growth, modeling, remodeling and fracture repair (Frost, 1983).

The forming and shaping of bone and maintenance and repair throughout life is carried out by the combined cellular efforts of bone modeling and remodeling. These two processes shape the skeleton and adapt it to the incoming load and strain by optimizing bone strength, minimizing mass and maintaining mechanical competence. Growth and fracture repair is largely outside the scope of the thesis and will not be tread upon, while modeling is briefly described, and remodeling gone through thoroughly.

Bone Modeling

Bone modeling is the physiological process by which the bones are shaped during development and adapted in response to physiological changes or external mechanical stimuli. The modeling process occurs mainly during growth.

Modeling incidences can occur as bone formation without prior bone resorption on the periosteal surfaces of bone to increase bone size and modify bone size and shape according to genetic programming or as an adaptation response to prevailing loading circumstances (Clarke, 2008; Martin and Seeman, 2008; Wang and Seeman, 2013). Importantly, during bone modeling, resorption and formation are processes occurring independently of each other at different locations, so the two are in essence uncoupled (Clarke, 2008). As modeling incidences are limited during adulthood, where remodeling is more prominent, the process is outside the scope of this thesis and will not be discussed further.
Bone Remodeling

Bone remodeling is a lifelong process and, on average, the entire skeleton is replaced every 10 years. The process involves replacing old bone with new to mend micro-fractures and maintain bone strength, as well as contributing to optimal control of mineral homeostasis, all processes important for maintaining a healthy skeleton. As bone ages, micro damage causes the bones to become fragile which compromises the weight bearing function of the skeleton. Bone remodeling secures adequate maintenance of bones ensuring their functional capacity (Seeman and Delmas, 2006).

Bone remodeling is taking place in both cortical and trabecular bone, however, the remodeling rates in the two compartments are quite different. The estimated remodeling rate in trabecular bone is 28% per year, while only being an estimated 4% per year in cortical bone (Manolagas, 2000).

Spatial orchestration of bone cells

The bone remodeling process is carried out by the coordinated efforts of a lineup of cells (osteoclasts, osteoblasts, osteocytes, and bone lining cells) which together comprise the basic multicellular units (BMUs)(Martin and Sims, 2005; Martin and Seeman, 2008; Parfitt, 2002; Sims and Gooi, 2008). Approximately 1-2 million active BMUs are estimated to be present at any given time in the human skeleton (Martin and Seeman, 2007). Bone remodeling in trabecular bone in humans, has been shown to take place in specialized bone remodeling compartments (BRCs) (Andersen et al., 2009; Hauge et al., 2001; Eriksen et al., 2007). In these compartments, a canopy of cells create an optimal microenvironment for bone remodeling, screening the remodeling site from the surrounding BM compartment, while perforating blood vessels supply progenitor cells and regulatory factors for the BRC (Andersen et al., 2009; Eriksen et al., 2007; Hauge et al., 2001).

The bone remodeling cycle

The remodeling process is carried out in a sequence of events involving the engagement from a quiescent phase to an activation phase followed by a resorption phase, reversal phase, formation phase, and termination phase (Parfitt, 2002; Parfitt, 2006; Seeman and Delmas, 2006). Normally, these events occur in a sequential manner where resorbed bone is always replaced by adequate amounts of new bone and complete replacement, a process referred to as coupling (Figure 2).

The bone remodeling cycle is started by the recruitment of osteoclast precursors to the site of remodeling. This is believed to involve signaling by apoptotic osteocytes and bone lining cells initiated by local micro damage (Burr, 2002; Henriksen et al., 2009b). The signaling is thought to be mediated by decreased transforming growth factor β (TGF-β) signaling from the osteocytes, which under normal circumstances inhibits osteoclastogenesis and bone resorption (Henriksen et al., 2009b). Osteocytes
were also recently shown to express Receptor Activator of Nuclear Factor κβ Ligand (RANKL), a key osteoclastogenic cytokine, potentially adding to the recruitment of osteoclast precursors to the site of remodeling (Xiong et al., 2011; Nakashima et al., 2011). These mechanisms cause osteocytes and bone lining cells to specifically target a local bone site for remodeling by attracting osteoclast precursors and stimulate osteoclastogenesis.

Following activation, bone lining cells remove a thin layer of matrix followed by retraction to make space for the osteoclasts (Everts et al., 2002; Henriksen et al., 2009b), and formation of the BRC by the bone lining cells. Osteocyte signaling recruits vessels to penetrate the BRC wall allowing access of bone cell precursors from the BM and circulation (Andersen et al., 2009; Eriksen et al., 2007). Osteoclast precursors summoned from the circulation or BM (Andersen et al., 2009; Parfitt, 2006; Sims and Gooi, 2008) are in the presence of RANKL differentiated into mature osteoclasts and initiate resorption, lasting 2-4 weeks for the individual osteoclast (Manolagas, 2000). As the osteoclasts complete resorption of the target site, they undergo apoptosis (Manolagas, 2000), likely induced by calcium or TGF-β released from the bone matrix (Houde et al., 2009; Lorger et al., 2000; Nielsen et al., 2007). The remodeling cycle enters a lag phase, called the reversal phase, where the osteoclasts are removed from the resorption pit, which is then prepared for bone formation. This phase involves the formation of a “cement line” separating old bone from new. The cellular origin of this line is currently not clear, but recent findings suggest that the presence of so-called “reversal cells” of osteoblastic origin might be involved in the initiation of the formation phase (Andersen et al., 2013). Also osteoclasts have been implicated in this process, by possibly depositing osteoblast targeted signals for regulation of bone formation (Everts et al., 2002; Karsdal et al., 2007; Karsdal et al., 2008). Osteoblast precursors are recruited to the site of bone remodeling and are differentiated into mature osteoblasts through mechanisms, which are still being investigated. Recruitment signals possibly consist of a mix of osteoclast and bone derived signals (Henriksen et al., 2009b; Henriksen et al., 2014; Karsdal et al., 2007; Karsdal et al., 2008; Kim et al., 2012; Lotinun et al., 2013; Martin et al., 2009; Martin and Sims, 2005; Pederson et al., 2008; Ryu et al., 2006; Takeshita et al., 2013). During the formation phase mature osteoblasts deposit osteoid, followed by layering of collagens and mineralization of the matrix, resulting in newly formed bone. The formation phase lasts 4-6 months, and after adequate amounts of bone have been formed, the cycle enters the termination phase, concluding the bone remodeling cycle. Osteoblasts either become bone embedded osteocytes, develop into bone lining cells, or undergo apoptosis (Henriksen et al., 2009b). The exact mechanism for this transition is not yet fully understood, but possibly includes the secretion of sclerostin by osteocytes, inhibiting further bone formation by the osteoblasts (Henriksen et al., 2009b; Poole et al., 2005).
Coupling in Remodeling and Between Bone Cells

In order for bone mass to remain relatively constant throughout life, the amount of bone formation must equal the amount of bone resorption during bone remodeling (Hattner et al., 1965; Takahashi et al., 1964). Since bone remodeling requires the combined efforts of both bone resorbing osteoclasts and bone forming osteoblasts, cross talk between the different cell types is necessary to control this process, also referred to as coupling (Howard et al., 1981; Sims and Walsh, 2012; Karsdal et al., 2007; Martin et al., 2009; Martin and Sims, 2005). It is important to emphasize that the strict coupling described here only occurs at the time of peak bone mass in adults. Earlier in life, the balance is dominated by a positive gain in bone, which is mainly mediated by modeling. After reaching peak bone mass, the balance shifts towards an overall negative bone balance for the remaining lifetime (Martin et al., 2009). Cells of the osteoblast lineage, from progenitor to osteocyte, produce stimulatory and inhibitory signals and factors that tightly regulate osteoclastogenesis function and activity; the most prominent probably being the osteoclastogenic factor RANKL and its soluble decoy receptor osteoprotegerin (Lacey et al., 1998; Simonet et al., 1997; Boyle et al., 2003). In addition the osteoclasts have also been shown to produce...
signals affecting the osteoblast recruitment, formation and activity, both through release of factors from the degraded bone and from the osteoclasts themselves. A large effort has been aimed at identifying osteoclast derived factors affecting osteoblasts and bone formation, for obvious therapeutic reasons, but the signaling mechanisms seem to be more complicated than initially thought, and not limited to a single molecule acting as master regulator (Henriksen et al., 2014; Karsdal et al., 2007; Karsdal et al., 2008; Kim et al., 2012; Lotinun et al., 2013; Martin and Seeman, 2008; Negishi-Koga et al., 2011; Pederson et al., 2008; Ryu et al., 2006; Takeshita et al., 2013; Walker et al., 2008; Zhao et al., 2006).

**Osteoporosis**

Changes in the delicate balance of bone remodeling can result in pathological conditions, such as for example osteoporosis, where changes in osteoclast and osteoblast activity lead to a general degradation of the skeleton; an effect often mediated by reductions in hormone production (Manolagas, 2000).

**Epidemiology and pathology**

Osteoporosis is a common disease affecting an estimated 12 million people in the US alone, and the disease accounts for roughly 1.5 million osteoporotic fractures in the US each year (U.S.Deptartment of Health and Human Services, 2004). With an aging population, the medical and socioeconomic repercussions will increase over time (Burge et al., 2007). The disease is characterized by a systemic loss of bone mass, strength, and micro architecture, which leads to an increased risk of fractures (NIH Consensus Development Panel on Osteoporosis Prevention, 2001). Bone mineral density (BMD) is commonly used as a hallmark for osteoporosis, and can be assessed with dual x-ray absorptiometry (DXA). Osteoporosis is defined by a T score of less than 2.5, meaning more than 2.5 standard deviations below the average of a young adult mean BMD (Kanis, 1994). There are a range of significant risk factors for the development of osteoporosis, the most prominent being loss of sex hormones during menopause but they also include age, diet, ethnicity, alcohol intake, smoking, genetic predispositions, immobility, and medication (Seeman, 2003). In most cases, osteoporosis is caused by a gradual unbalancing of the bone remodeling process, where bone resorption exceeds bone formation resulting in a net bone loss (Drake and Khosla, 2013). This change in remodeling is in itself a naturally occurring process caused by aging. The estrogen deficiency occurring in postmenopausal women, however, has a profound effect on the balancing of bone resorption and bone formation, leading to increased osteoclastogenesis, increase in osteoclast activity, and osteoclast longevity (Drake and Khosla, 2013).
Different treatments modalities are available for osteoporosis, such as anti-resorptive- or anabolic treatments, or dietary supplements such as Vitamin D and calcium. The anti-resorptive drugs are currently the most widely used treatments for osteoporosis and, as the name indicates, they focus on removing the osteoclasts or inhibiting their ability to resorb the bone matrix. Anti-resorptives very efficiently lower bone resorption and this results in significant reduction of fracture rate in patients. However, secondary to their anti-resorptive effect is a decrease in bone formation which limits their overall effect. The anti-resorptives include bisphosphonates (BPs), oestrogen and selective oestrogen receptor modulators (SERMs), and Denosumab, a neutralizing RANKL antibody, as well as calcitonin and strontium renate (McClung et al., 2006; Papapoulos, 2013; Russell et al., 2008; Watts, 2013; Cummings et al., 2009; Rogers et al., 2011; Delmas et al., 1997; Ettinger et al., 1999; Chesnut, III et al., 2000; Meunier et al., 2004). Parathyroid hormone (PTH), or active fragments here of, are currently the only treatment increasing bone formation, however, besides the effect on osteoblasts is a general increase in bone turnover actively increasing bone resorption secondary to bone formation, limiting its overall potential (Cosman and Greenspan, 2013; Neer et al., 2001). An overview of the different treatments is given in Table 1. Overall the current treatments suffer from the coupling between resorption and formation which limits their overall efficacy. New treatments may be targeted at osteoclast function while preserving bone formation, or by activating osteoblasts in face of normal bone resorption (Thudium et al., 2012; Eisman et al., 2011; McClung et al., 2014).

Table 1. Osteoporosis treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Example</th>
<th>Target</th>
<th>OCL→OB coupling</th>
<th>Change in formation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current treatments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bisphosphonate</td>
<td>Alendronate</td>
<td>Bone resorption</td>
<td>Yes</td>
<td>Decreased</td>
<td>(Liberman et al., 1995)</td>
</tr>
<tr>
<td>α-RANKL</td>
<td>Denosumab</td>
<td>Bone resorption</td>
<td>Yes</td>
<td>Decreased</td>
<td>(McClung et al., 2006)</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Miacalcin</td>
<td>Bone resorption</td>
<td>Yes</td>
<td>Decreased</td>
<td>(Sexton et al., 1999; Chesnut, III et al., 2000)</td>
</tr>
<tr>
<td>SERMs</td>
<td>Raloxifene</td>
<td>Bone resorption</td>
<td>Yes</td>
<td></td>
<td>(Delmas et al., 1997; Ettinger et al., 1999)</td>
</tr>
<tr>
<td>Strontium Ranelate</td>
<td>Protelos</td>
<td>Bone resorption/bone formation</td>
<td></td>
<td>(Increased)</td>
<td>(Meunier et al., 2004)</td>
</tr>
<tr>
<td>PTH (or fragments)</td>
<td>Teriparatide</td>
<td>Bone formation/bone resorption</td>
<td>Yes</td>
<td>Increased</td>
<td>(Neer et al., 2001)</td>
</tr>
<tr>
<td>In development</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathepsin K inhibitor</td>
<td>Odanacatib</td>
<td>Bone resorption</td>
<td>(Yes)</td>
<td>Decreased</td>
<td>(Eisman et al., 2011)</td>
</tr>
<tr>
<td>α-Sclerostin</td>
<td>Romosozumab</td>
<td>Bone formation</td>
<td>No (modeling)</td>
<td>Increased</td>
<td>(McClung et al., 2014)</td>
</tr>
</tbody>
</table>
Osteoclasts

Osteoclasts are unique cells in that they are the only cells responsible for the resorption of bone. In light of this fact, the correct functionality and regulation of the osteoclast is crucial for maintaining bone homeostasis.

Hematopoiesis

Osteoclasts are derived from hematopoietic stem cells (HSCs) which represent a rare population of cells with the potential of long-term self renewal and a capacity for multi lineage differentiation (Figure 3). This process leads to the replenishment of billions of new blood cells for the human body every day (Ema et al., 2005; Ogawa, 1993). HSCs mainly reside in the BM and are primarily present in quiescent state in human adults. HSCs are capable of either self renewal through symmetric division giving rise to two identical progeny cells or through asymmetric division giving rise to two different daughter cells that can either sustain long-term hematopoiesis or act as progenitor cell for the distinct blood lineages (Morrison and Kimble, 2006). The fate of the HSC is believed to be directed by a combination of different stochastic events during division and extracellular signals from the HSC niche (Enver et al., 1998; Metcalf, 1998; Morrison and Weissman, 1994; Ogawa, 1999). Hematopoietic stem cells have a very low representation in blood and BM and because no single marker identifying this population is known, generally a combination of markers/antibodies is used to identify and describe purified stem- and progenitor cell populations, such as cluster of differentiation (CD). CD34 is expressed on the surface of human progenitor and HSCs regardless of cell cycle status, and this marker is often used to enrich human HSCs (Moore, 2009; Sidney et al., 2014). The hematopoietic nature of CD34+ cells make them rare in the peripheral blood and they are instead often isolated from the BM or umbilical cord blood or through mobilization into peripheral blood (Civin et al., 1984; Gianni et al., 1989).
Figure 3. An overview of the hematopoietic hierarchy.
Hematopoiesis comprises the process in which HSCs residing in the bone marrow (BM) give rise to all types of mature blood cells in the peripheral blood (PB). Osteoclasts are differentiated from progenitors of the monocytic lineage. 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).

Osteoclastogenesis

Mature osteoclasts are large multinucleated cells which originate from the hematopoietic stem cell (HSC) and which are formed through fusion of mononuclear precursors in a process known as osteoclastogenesis. During osteoclastogenesis, HSCs differentiate into common myeloid progenitor cells and then into CD14 expressing monocytes directed by the presence of a range of specific cytokines and the expression of transcription factors such as PU.1. (Shalhoub et al., 2000; Way et al., 2009; Tondravi et al., 1997). PU.1 deficient mice lack both macrophages and osteoclasts emphasizing its importance in osteoclastogenesis (Tondravi et al., 1997). Two cytokines are crucial for osteoclast differentiation, namely macrophage colony stimulating factor (M-CSF) and RANKL. M-CSF acts on proliferation and survival
of the monocytes (Felix et al., 1990) and induces the expression of receptor activator of nuclear factor κB (RANK) on the surface of maturating osteoclasts (Arai et al., 1999). Binding of RANKL to RANK activates a number of intracellular pathways, of which nuclear factor κB (NFκB), c-fos, nuclear factor activated T cells c1 (NFATc1), and TNF receptor associated factor 6 (TRAF6) are the most prominent and required for osteoclastogenesis (Wang et al., 1992; Lacey et al., 1998; Li et al., 2000; Zhao et al., 2007). The importance of the RANK/RANKL interaction is underlined by the complete lack of osteoclasts in RANK knock-out (KO) mice (Dougall et al., 1999), and development of infantile malignant osteopetrosis in patients with homozygous mutations in RANK and RANKL (Guerrini et al., 2008; Sobacchi et al., 2007). Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL and acts as a negative regulator of osteoclastogenesis and the overall osteoclast differentiation is dictated by the RANKL/OPG ratio (Simonet et al., 1997). Committed osteoclast precursors will start to fuse, a process which is regulated by dendritic cell-specific transmembrane protein (DC-STAMP) (Yagi et al., 2005). As pre-osteoclasts fuse, they start expressing a range of proteins, such as tartrate resistant acid phosphatase (TRAP), cathepsin K, Chloride channel 7 (ClC-7), and the osteoclastic vacuolar H⁺-ATPase (V-ATPase), including its subunit T-cell immune regulator 1 (TCIRG1)(also referred to as the a3 subunit), which are important for correct osteoclast resorptive function (Figure 4)(Burstone, 1959; Kornak et al., 2000; Kornak et al., 2001; Gowen et al., 1999).

Figure 4. Osteoclastogenesis.

Lineage commitment

Hematopoietic stem cell (CD34+)

Multipotent progenitor

Monocyte (CD14+)

PU.1

CSF1R

c-Fos

c-Fms

RANK

NFκB

TRAF6

NFATc1

TRAP

DC-STAMP

OPG

M-CSF

RANKL

Pre-osteoclast

Fused polykaryon

c-Src

αβ3

MITF

NFATc1

Cathepsin K

V-ATPase (a3)

CIC-7

OSTM1

AEZ

CAl

CalcitoninR

Mature osteoclast

um>3221</RecNum><IDText>Lowve from HSCs. Commitment to the osteoclast lineage involves differentiation of monocytes through pre-osteoclasts, fusion of pre-osteoclasts, and polarization of mature osteoclasts. A number of transcription factors and cytokines direct the expression of multiple proteins needed for proper differentiation and function of the osteoclasts. Inspired by (Boyle et al., 2003; Mellis et al., 2011).

23
Bone Resorption

The osteoclast is unique in its capacity to dissolve the inorganic and organic part of the bone matrix (Blair et al., 1986; Baron et al., 1985), processes which require a range of osteoclast specific proteins and functions. In addition, the degradation process depends upon cytoskeletal rearrangements leading to the formation of a specialized compartment between the osteoclast and the bone crucial for bone resorption (Zou and Teitelbaum, 2010). A schematic overview of a bone attached osteoclasts is given in Figure 5.

At osteoclast maturation, cytoskeletal changes in the osteoclast lead to polarization of the cell and the formation of a sealing zone which defines a closed space between the osteoclast and the bone called the resorption lacunae (Vaananen and Horton, 1995). The function of the sealing zone is to create an environment closed off from the immediate extracellular milieu, which functions like an “extracellular lysosome”. The sealing zone is formed by the assembly of an intracellular actin ring composed of F-actin fibers (Vaananen and Horton, 1995; Lakkakorpi et al., 1989). Upon attachment to the bone surface, the tight binding of the sealing zone is mediated by the osteoclast cell surface protein αvβ3-integrin and also requires the signal transducers c-src, Syk, and proline-rich tyrosine kinase 2 (Boyle et al., 2003; Teitelbaum, 2007; Zou et al., 2007).

In vivo studies in mice lacking the β3 part of the receptor have shown that osteoclasts are formed but mice become osteopetrotic due to osteoclast dysfunction (McHugh et al., 2000). Within the sealing zone, the osteoclast generates a highly folded part of the plasma membrane called the ruffled border (membrane). This ruffled border is formed by the fusion of intracellular acidic vesicles with the plasma membrane bounded by the sealing zone (Teitelbaum, 2007). Active bone resorption is taking place in the resorption lacuna below the ruffled border. The capacity of the mature osteoclast to resorb bone depends on the secretion of a series of ions and enzymes. Dissolution of the inorganic matrix is mediated through acidification of the resorption lacunae (Baron et al., 1985). This acidification process involves proton transporting V-ATPases, including TCIRG1, brought to the ruffled border via vesicle fusion, which then transports protons across the ruffled border (Baron et al., 1985; Frattini et al., 2000; Kornak et al., 2000; Toyomura et al., 2000; Blair et al., 1989). In order to maintain electroneutrality, the proton secretion is balanced by transport of chloride ions through the chloride anti-porter ClC-7 (Kornak et al., 2001; Kasper et al., 2005; Schaller et al., 2005; Schlesinger et al., 1997; Henriksen et al., 2004). The transport of electrolytes through the V-ATPase and ClC-7 results in secretion of hydrochloric acid into the resorption lacunae, creating an acidic environment of approximately pH 4.5. The low pH is crucial for dissolution of the inorganic matrix and proteolytic enzyme activity (Blair et al., 1989; Baron et al., 1985; Kornak et al., 2001). Pathological mutations in the V-ATPase or ClC-7 can lead to severe osteopetrosis in both mice and humans due to lack of bone resorption by the osteoclasts (Table 2)(Del Fattore et al., 2006; Frattini et al., 2000; Kornak et al., 2001).
Protons needed for transport via the V-ATPase are provided by the intracellular enzyme carbonic anhydrase II (CAII), which catalyses the conversion of CO$_2$ and H$_2$O into H$_2$CO$_3$, and further hydrolyzed into H$^+$ and HCO$_3^-$ (Gay and Mueller, 1974; Henriksen et al., 2008). Patients lacking CAII function develop osteopetrosis from loss of osteoclast function (Sly et al., 1983). HCO$_3^-$ is then exchanged with chloride ions through the anion exchanger 2 (AE2) in the basolateral membrane, ensuring continued availability of ions for ongoing acidification and bone resorption (Teti et al., 1989).

The acidification of the resorption lacunae mobilizes the inorganic part of the bone exposing the collagen rich organic matrix (Nielsen et al., 2007). The exposed collagen matrix is then degraded by proteases released into the resorption lacunae (Henriksen et al., 2006; Delaisse et al., 2003; Gelb et al., 1996). The most prominent enzyme in this regard is the lysosomal cysteine protease cathepsin K, which is secreted from the ruffled border and activated in the acidic environment (Lecaille et al., 2008). Upon activation, cathepsin K efficiently cleaves collagen type I. Failure to produce functional cathepsin K due to mutations in the **CTSK** gene encoding cathepsin K results in the bone disease pycnodysostosis (Gelb et al., 1996), characterized by impaired degradation of the collagen matrix leading to osteosclerosis of the bone, skull deformities, short stature and weak bones with increased fracture rate (Fratzl-Zelman et al., 2004; Gowen et al., 1999; Gelb et al., 1996).

The role of matrix metallo-proteases (MMPs) in bone degradation is not completely clear (Delaisse et al., 2003; Everts et al., 1993; Henriksen et al., 2012). Studies have shown that MMPs might participate in bone degradation. However, under normal circumstances MMP activity seems to be negligible compared to cathepsin K, and restricted to specific skeletal sites (Delaisse et al., 2003; Henriksen et al., 2012). The exact protease profile of osteoclasts appears to be at least partly dependent on the bone type that is being resorbed, such flat bones or long bones, and context, such as in pathological disease states. This can also be referred to as osteoclast subtype (Henriksen et al., 2011).

Products generated through bone resorption are endocytosed by the osteoclast, transported through the cell and released through the secretory domain opposite the osteoclasts resorptive domain (Nesbitt and Horton, 1997). After a number of resorptive cycles, the osteoclast undergoes apoptosis; a process likely induced by the release of calcium and TGF-β from the resorbed matrix (Houde et al., 2009; Hughes et al., 1996; Lorget et al., 2000; Nielsen et al., 2007).
Figure 5. Schematic drawing of the bone resorbing osteoclasts.
After attachment to the bone surface through integrin binding, the osteoclast secretes acid into the resorption lacuna through the coordinated efforts of the osteoclastic V-ATPase and the chloride channel CIC-7. This process ensures breakdown of the inorganic matrix and is followed by the secretion of the collagen degrading proteinase cathepsin K. Protons and chloride ions needed for proper acidification are provided by the membrane protein AE2 and the enzyme CAII. CIC-7, chloride channel 7; CAII, carbonic anhydrase; AE2, anion exchanger 2.
Osteoblast Lineage Cells

The other key player in bone remodeling is the osteoblast. The osteoblasts are mononuclear bone cells responsible for bone formation and derive from multipotential mesenchymal stem cells (MSCs) located in the BM (Capulli et al., 2014). Cells of the osteoblast lineage exist as three functionally different cell types in the skeleton: osteoblasts, osteocytes, and bone lining cells. The differentiation of the MSCs into osteoblast lineage cells is controlled by a range of hormones, cytokines and corresponding receptors which regulate the expression of transcription factors needed for correct lineage differentiation.

Maybe the most important role of the osteoblast is the formation of new bone. Bone formation by osteoblasts can be divided into three phases: I) a proliferation phase involving biosynthesis of the extracellular components, II) development and organization of the extracellular matrix, and III) extracellular matrix mineralization. The process itself includes the deposition of osteoid layer consisting mainly of collagen type I and bone specific proteins. Osteoid deposition is followed by mineralization where hydroxyapatite crystals are deposited in the collagen framework (Capulli et al., 2014). Morphology and expression of mRNA and proteins vary greatly in response to the requirements of the different phases of bone formation. After bone formation is completed, the osteoblasts either become embedded in the bone as osteocytes, turn into bone lining cells, or die through apoptosis (Manolagas, 2000; Aubin, 2001; Capulli et al., 2014).

In addition to bone formation, a secondary role for the osteoblast lineage cells is the production of cytokines regulating osteoclastogenesis; the most important being RANKL and its soluble decoy receptor OPG. The expression is thought to be mainly isolated to osteoblast precursors and terminally differentiated osteocytes (Nakashima et al., 2011). The production of cytokines is in turn regulated by hormones such as PTH, Vitamin D, and estrogen, as well as cytokines such as TGF-β, but the regulatory mechanisms are complex and still under investigation.

Osteocytes are cells derived from osteoblasts embedded in the bone following bone formation (Manolagas, 2000). Osteocytes are the most abundant bone cell in the body, mainly due to its long lifespan, estimated to be around 25 years (Knothe Tate et al., 2004). The osteocytes are morphologically characterized by having dendritic cytoplasmic extensions connecting them to each other and the bone surface allowing them to survive and signal from within the bone matrix. The general function of the
osteocyte is thought to include mechanosensory activity actively directing the actions of osteoblasts and osteoclasts within the BMU, in response to external physiological stress (Manolagas, 2000; Bonewald, 2011). In addition, osteocytes are believed to direct the targeted remodeling happening in older bones and in response to microcracks; a signal which is mediated through the apoptosis of the osteocyte and sclerostin signaling (Bonewald, 2011; Sims and Vrahnas, 2014).

The thin layer of cells covering all quiescent bone surfaces is called bone lining cells and also derive from the osteoblast lineage (Manolagas, 2000; Miller et al., 1989). Here they form a barrier between the BM and the bone matrix. The bone lining cells are believed to be important for the process of targeted bone remodeling (Everts et al., 2002; Andersen et al., 2013).
Osteopetrosis

Osteopetrosis is a heterogenous group of rare genetic diseases characterized by either lack of osteoclasts or defects in bone resorption by osteoclasts (Balemans et al., 2005; Bollerslev et al., 1993; Del Fattore et al., 2006; de Vernejoul and Benichou, 2001; Del Fattore et al., 2008; Tolar et al., 2004). Its pathological impact aside, investigation of patients and mice with osteopetrosis have provided invaluable insight into how osteoclasts function and how bone cells communicate with each other.

Pathogenesis

Radiographic findings in a patient with increased bone density were first described by Albers-Schönberg (Albers-Schönberg, 1904) and since then a number of different osteopetrosis forms have been described, ranging from mild or asymptomatic such as autosomal dominant osteopetrosis (ADO), to very severe or lethal as in autosomal recessive osteopetrosis (ARO), often referred to as infantile malignant osteopetrosis (IMO).

The disease presents with a general increase in skeletal bone mass due to increased density of the bone. Radiological findings in different forms of osteopetrosis can display as evident sclerostation of the bone, often with a bone-in-bone appearance in phalanges, long bones and pelvic bones, all varying in severity dependent on type (Balemans et al., 2005; Shapiro, 1993; Loria-Cortes et al., 1977; Tolar et al., 2004). The sclerotic bones are brittle and prone to fractures, a phenotype that is probably attributed to the congenital developmental nature of the disease (Neutzsky-Wulff et al., 2010).

Osteopetrosis is often associated with compression of the cranial nerves due to bone thickening at the base of the skull, which can cause visual impairment and hearing loss (Sobacchi et al., 2013). In addition, patients suffer from disturbed tooth eruption, as osteoclasts are normally responsible for resorbing a path through the jaw for the developing tooth (Helfrich, 2005).
Table 2. Mutations leading to osteopetrosis

<table>
<thead>
<tr>
<th>Protein (Gene)</th>
<th>Disease</th>
<th>Function</th>
<th>Osteoclast/Osteoblast profile</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCIRG1/a3 (TCIRG1)</td>
<td>ARO</td>
<td>Acidification</td>
<td>Increased osteoclast number Normal/increased bone formation</td>
<td>(Kornak et al., 2000; Frattini et al., 2000)</td>
</tr>
<tr>
<td>CLC-7 (CLCN7)</td>
<td>ARO/ADO</td>
<td>Acidification</td>
<td>Increased osteoclast number Normal/increased bone formation</td>
<td>(Kornak et al., 2001; Frattini et al., 2003; Cleiren et al., 2001; Campos-Xavier et al., 2003)</td>
</tr>
<tr>
<td>OSTM1/ (OSTM1)</td>
<td>ARO</td>
<td>Acidification</td>
<td>Increased osteoclast number Normal/increased bone formation</td>
<td>(Chalhoub et al., 2003; Pangrazio et al., 2006)</td>
</tr>
<tr>
<td>SNX10/ (SNX10)</td>
<td>ARO</td>
<td>Endosomal/vesicular trafficking</td>
<td>Unknown</td>
<td>(Pangrazio et al., 2013)</td>
</tr>
<tr>
<td>RANKL/ (TNFSF11)</td>
<td>ARO</td>
<td>Osteoclastogenesis</td>
<td>No osteoclasts Decreased bone formation</td>
<td>(Sobacchi et al., 2007)</td>
</tr>
<tr>
<td>RANK/ (TNFRSF11A)</td>
<td>ARO</td>
<td>Osteoclastogenesis</td>
<td>No osteoclasts Decreased bone formation</td>
<td>(Guerrini et al., 2008; Pangrazio et al., 2012)</td>
</tr>
<tr>
<td>cathepsin K/ (CTSK)</td>
<td>Pycnodysostosis</td>
<td>Proteolysis</td>
<td>Increased osteoclast number osteoblast phenotype unclear</td>
<td>(Gelb et al., 1996)</td>
</tr>
</tbody>
</table>

Current Treatment for Osteopetrosis

While mild forms of osteopetrosis can go undetected for many years, ARO is a fatal disease and without hematopoietic stem cell transplantation the disease is lethal before the age of 6 years. Often the lethal outcome is caused by pancytopenia and recurrent infections which are treated with transfusions and antibiotics to alleviate the symptoms (Fasth and Porras, 1999; Wilson and Vellodi, 2000). The first BM transplantations of osteopetrotic children were performed in the seventies (Ballet et al., 1977; Coccia et al., 1980) after transplantation studies had shown permanent cure of osteopetrotic gl/gl and microthalamic (mi/mi) mice (Walker, 1975a; Walker, 1975b). Today BM transplantations have been applied in a number of patients and the long term clinical outcome evaluated (Driessen et al., 2003; Fasth and Porras, 1999; Gerritsen et al., 1994; Sobacchi et al., 2013).

The outcome of BM transplantation is highly dependent on human leukocyte antigen (HLA) matching, and the age of the patient at transplantation (Sobacchi et al., 2013). Studies have shown that transplantations performed before the age of 10 months increase the chance of full engraftment, while transplantations performed at older age increase the risk of graft rejection or autologous reconstitution (Sobacchi et al., 2013). Transplantations performed in patients older than 2 years markedly increase the risk of hypercalcemia and the risk of neuronal damage from compression of the skull (as reviewed in (Askmyr et al., 2009a; Sobacchi et al., 2013). This is in line with transplantation studies in mice showing that the osteopetrotic phenotype in oc/oc
mice can be reversed using non-ablative neonatal transplantation of healthy donor cells (Flores et al., 2010). Overall, BM transplantation is an effective therapy for treatment of osteopetrosis if initiated early. However, in cases where no HLA matched donors can be found, there is a clear need for alternative treatment options, such as gene therapy.

Osteoclast-rich Osteopetrosis

The severe form of osteopetrosis, ARO, is a rare recessive disorder affecting approximately 1 in 200,000-300,000 (Balemans et al., 2005). ARO is most often caused by homozygous or compound heterozygous mutations in one of the two genes, \textit{TCIRG1} or \textit{CLCN7}, resulting in ablated osteoclast function, but not number and is therefore referred to as an osteoclast-rich osteopetrosis (Figure 6)(Del Fattore et al., 2006; Frattini et al., 2000; Frattini et al., 2003; Kornak et al., 2000; Kornak et al., 2001; Segovia-Silvestre et al., 2008; Sobacchi et al., 2001; Sobacchi et al., 2013). Mutations in \textit{CLCN7} can also lead to autosomal dominant osteopetrosis (ADO) and intermediate autosomal recessive osteopetrosis (IARO), with an osteoclast-rich cellular phenotype although the disease manifestation in both cases is generally more benign than the homozygous form. Mutations in the genes encoding osteopetrosis-associated transmembrane protein 1 (OSTM1), sorting nexin 10 (SNX10) and Plexstrin homology domain-containing family M member 1 (PLEKHM1), are also described as being osteoclast-rich forms of osteopetrosis, but will not be mentioned further in this thesis, as they are relatively poorly understood, and only few cases have been identified (Aker et al., 2012; Megarbane et al., 2013; Pangrazio et al., 2006; Pangrazio et al., 2013; Van et al., 2007). An overview of the different mutations is given in Table 2

Osteoclast-rich osteopetrosis is characterized by an increased number of non-resorbing osteoclasts that may result in osteosclerosis of the bone and abnormal BM cavity development which limits hematopoiesis. Despite reduced bone resorption, bone formation is ongoing leading to large increases in bone mass further worsening the pathology (Bollerslev and Andersen, Jr., 1988; Del Fattore et al., 2006; Frattini et al., 2000; Kornak et al., 2000; Kornak et al., 2001). Although bone mass is increased, the bones are brittle and prone to fracture.
TCIRG1 deficiency

TCIRG1 is a subunit of the osteoclast vacuolar type H\(^+\)-ATPase (V-ATPase) encoded by the \textit{TCIRG1} gene. The a3 subunit is a 7 transmembrane domain protein and is a member of the V-ATPase a-subunit family. The subunit is primarily expressed in osteoclasts, where it is crucial for functional bone resorption (Manolson et al., 2003).

V-ATPases in general are important for regulation of pH within many intracellular compartments, such as lysosomes, endosomes, and secretory vesicles (hence the name “vacuolar”), and does so by ATP-driven transportation of H\(^+\) ions across the plasma membrane (Sun-Wada and Wada, 2013). The V-ATPase itself is a large multisubunit/multidomain membrane localized protein consisting of two subdomains, a structural V\(_0\) domain and an enzymatic V\(_1\) (Jefferies et al., 2008). A schematic overview of the V-ATPase is given in Figure 7.

Pathological mutations in the \textit{TCIRG1} gene lead to defective acidification and lack of bone resorption in both human patients and animal models (Blair et al., 2004; Del Fattore et al., 2006; Frattini et al., 2000; Kornak et al., 2000; Moscatelli et al., 2013; Taranta et al., 2003). Mutations in \textit{TCIRG1} accounts for nearly 50% of all ARO cases, making this the most frequent cause of osteopetrosis (Del Fattore et al., 2008; Sobacchi et al., 2001; Sobacchi et al., 2013). Much of the current knowledge about the V-ATPase and its role in osteoclasts are derived from extensive studies in osteopetrotic patients and mouse models bearing mutations in \textit{TCIRG1}.

The first connection between a proton pump gene and osteopetrosis was indicated by studies in the spontaneous osteopetrotic \textit{oc/oc} mouse model (Marks, Jr. et al., 1985; Seifert and Marks, Jr., 1985; Frattini et al., 2000; Kornak et al., 2000). Multiple
mutational studies led to the identification of the \textit{TCIRG1} gene encoding the a vacuolar proton pump subunit as a cause of osteopetrosis development in both humans and mice (Frattini et al., 2000; Kornak et al., 2000; Li et al., 1999). In humans, mutations are variable and include deletions, insertions, missense mutations and splice site mutations (Sobacchi et al., 2001; Del Fattore et al., 2006; Del Fattore et al., 2008). The main cellular phenotype in patients with mutations in \textit{TCIRG1} is the higher amount of non-resorbing osteoclasts found in bone biopsies compared to osteoclasts in healthy individuals (Flanagan et al., 2000; Frattini et al., 2000; Taranta et al., 2003). Osteoclasts from ARO patients are larger in size and generally display with more nuclei, but otherwise act phenotypically as normal osteoclasts in terms of attachment to bone and polarization (Taranta et al., 2003). In \textit{vitro} studies of osteoclasts with mutations in the \textit{TCIRG1} gene show that despite normal development, the lack of proton secretion abolish acid secretion (Blair et al., 2004), limiting the capacity of osteoclasts to resorb bone (Del Fattore et al., 2006; Moscatelli et al., 2013; Taranta et al., 2003). Despite lack of bone resorption, these patients show increased bone formation markers and, in alignment with this, a correlation has been shown between the number of non-resorbing osteoclasts and the number of osteoblasts \textit{in vivo} (Del Fattore et al., 2006; Taranta et al., 2003). In summary, these findings are indicative of ongoing bone formation in osteoclast-rich osteopetrosis despite the lack of bone resorption. The increased bone formation might also explain the severity of the phenotype compared to other types of osteopetrosis. From a therapeutic perspective recent studies in the \textit{TCIRG1} mutated oc/oc mouse using neonatal transplantation of healthy or gene corrected precursor cells indicate that the bone phenotype can be corrected using hematopoietic stem cell targeted gene therapy(Flores et al., 2010; Johansson et al., 2007).
Figure 7. Structural overview of the osteoclastic V-ATPase
The V-ATPase transports protons across the ruffled border, driven by the enzymatic conversion of ATP to ADP. This maintains the low pH in the resorption lacuna essential for proper bone resorption. Adapted from (Jefferies et al., 2008). a3 – TCIRG1.

ClC-7 deficiency

ClC-7 is a homodimeric membrane protein encoded by the CLCN7 gene. It is expressed in the lysosomes and at the ruffled border of mature osteoclasts where it functions as a voltage gated Cl⁻/H⁺ exchanger transporting chloride ions into the resorption lacuna. This transport actively balances the conductance generated by the proton flow during bone resorption, as described previously described (Henriksen et al., 2004; Henriksen et al., 2008; Henriksen et al., 2009a; Neutzsky-Wulff et al., 2008; Schaller et al., 2005). The importance of ClC-7 in bone resorption is highlighted by the fact that mutations in ClC-7 lead to osteopetrosis in humans, such as ARO, ADO and IARO, and in mice as seen in the ClC7-/- mouse or the knockin ADOII mice (Alam et al., 2014; Bollerslev et al., 1989; Frattini et al., 2003; Kornak et al., 2001; Neutzsky-Wulff et al., 2008; Cleiren et al., 2001). At the membrane, the ClC-7 homodimer binds to OSTM1, an interaction which is critical for stabilization of the channel and protection from degradation (Lange et al., 2006; Leisle et al., 2011) and lack of OSTM1 leads to osteopetrosis in both mice and humans (Chalhoub et al., 2003).

Patients with mutations in what was most likely ClC-7 were first described by Albers Schönberg in the early 20th century (Albers-Schönberg, 1904). In contrast to osteopetrosis originating from TCIRGI mutations, ClC-7 derived osteopetrosis exists in two types; the severe recessive form ARO, and the more benign dominant forms ADO and IARO, as previously mentioned; of which the later will not be discussed.
Homozygous or compound heterozygous mutations in ClC-7 give rise to autosomal recessive osteopetrosis due to acid secretion failure and the phenotype is similar to osteopetrosis caused by TCIRG1 mutations. Mutations in ClC-7 account for approximately 15% of ARO cases (Frattini et al., 2003; Kornak et al., 2001). These patients, in addition to the severe bone phenotype, suffer from primary neuronal defects leading to neuronal storage disease and retinal atrophy, an effect accounted to the secondary expression of ClC-7 in brain tissue (Frattini et al., 2003; Kasper et al., 2005; Kornak et al., 2001). This is in contrast to patients with TCIRG1 mutations where neuronal damage, such as blindness is most often caused by compression of the cranial nerve (Fasth and Porras, 1999). The cellular phenotype in patients with ARO due to ClC-7 mutations is very similar to the one seen in patients with TCIRG1 mutations.

Although not being characterized as ARO, the milder ADO has contributed greatly to expanding the knowledge on the underlying osteoclast pathology and mechanisms of osteoclast-rich osteopetrosis. In contrast to ARO, ADO is a heterogenous disease in terms of severity, and the penetrance has been estimated to be around 66% of carriers. This heterogeneity can most likely be attributed to the dimeric nature of ClC-7. Some carriers show no visual signs of disease except for mild increases in BMD, but the phenotypic penetrance varies greatly between patients (Benichou et al., 2000; Waguespack et al., 2007; Frattini et al., 2003; Cleiren et al., 2001), and seem to also correlate with osteoclast resorptive function in vitro (Chu et al., 2006). ADO is the most common form of osteopetrosis with an estimated prevalence estimated to be 5.5:100,000, however a more conservative guess is around 1:100,000 (Benichou et al., 2000).

Some of the clinical manifestations observed in ADO patients include delayed fracture healing (Bollerslev, 1989; Benichou et al., 2000; Waguespack et al., 2007), cranial neuropathies, and osteomyelitis (Waguespack et al., 2007).

Studies of the cellular phenotype in bone biopsies from ADO patients show high numbers of abnormally large TRAP positive osteoclast on the inner bone surface (Bollerslev et al., 1993), due to increased survival of osteoclasts not resorbing bone (Nielsen et al., 2007). In correlation with these findings, high levels of TRAP activity have been found in serum of ADO patients (Alatalo et al., 2004; Bollerslev and Andersen, Jr., 1988; Waguespack et al., 2002). The osteoclasts show no ruffled border formation and have a high number of intracellular vesicles, which correlates well with studies indicating that functional chloride transport is important for vesicular trafficking (Blair et al., 2004; Del Fattore et al., 2006; Kornak et al., 2001; Novarino et al., 2010; Sobacchi et al., 2013). ADO subjects have also shown increased eroded areas but non-significant decreases in resorption rate, indicating
reduced resorption capability of these osteoclasts (Bollerslev et al., 1993). In addition, mineral apposition rate (MAR) was increased in these patients while osteoblast parameters were unchanged compared to control subjects (Bollerslev et al., 1989). From a functional perspective, osteoclasts from ADO patients and ClC-7 deficient mice have reduced or no capability of extracellular acidification in vitro (Henriksen et al., 2009a; Neutzsky-Wulff et al., 2008).

Osteoclast-poor Osteopetrosis

Recent studies have identified rare cases of osteopetrosis where mutations lead to a complete absence of osteoclasts in vivo, described in the literature as an osteoclast-poor osteopetrosis (Guerrini et al., 2008; Sobacchi et al., 2007; Pangrazio et al., 2012). These patients have a milder phenotype development than classical ARO despite having no bone resorption due to their lack of osteoclasts. The difference in phenotype acceleration is suspected to be caused by the lack of anabolic signaling from osteoclasts.

RANKL deficiency

The absence of osteoclasts in a group ARO patients indicated mutations in the cell differentiation process and not the bone resorptive function itself. Mutations were identified in the \textit{TNFSF11} gene encoding the osteoclast master regulator cytokine RANKL. These patients displayed with a severe ARO phenotype resembling that of TCIRG1- or ClC-7 derived osteopetrosis but the phenotype developed at a slower pace (Sobacchi et al., 2007). The cellular phenotype display with a general lack of mature osteoclasts. Due to the non-cell autonomous origin of the RANKL mutation, which is usually expressed by non-hematopoietic stromal cells in the BM or osteocytes, these patients did not rescue by BM replacement (Sobacchi et al., 2007). Interestingly, isolated monocytes readily differentiated into multinucleated resorbing osteoclasts in vitro, in the presence of exogenous RANKL, corroborating the involvement of a RANKL mutation in these patients (Sobacchi et al., 2007).

RANK deficiency

Another subset of patients displaying with ARO phenotype similar to patients with RANKL mutations, and without osteoclast presence was found to bear mutations in \textit{TNFRSF11A} encoding RANK (Guerrini et al., 2008; Pangrazio et al., 2012). In addition to the normal ARO symptoms, these patients also suffer from hypogammaglobulinemia, indicating that RANK also plays a role in the development of the immune system (Guerrini et al., 2008; Pangrazio et al., 2012). This is also evident by the fact that lymph node development is hampered in RANK KO mice (Dougall et al., 1999). The cellular phenotype in patients is very comparable to that
of patients with RANKL mutations in that osteoclasts are absent as a result of disrupted RANK/RANKL signaling. In addition, very little or no osteoblasts, and consequently bone formation, is observed in biopsies from these patients (Pangrazio et al., 2012). In contrast to RANKL derived osteopetrosis, osteoclast precursors from RANK KO mouse are different, in that they do not differentiate into osteoclasts when RANKL is added in vitro, in line with the cell-autonomous nature of the mutation (Villa et al., 2009). As with the osteoclast-rich osteopetrosis, the findings in mouse models of osteoclast-poor osteopetrosis recapitulate the phenotypes observed in human. Namely that lacking osteoclasts, and, thus, osteoclast-secreted anabolic factors, leads to a reduction in bone formation and a less severe bone phenotype compared to its osteoclast-rich counterpart, supporting the view that osteoclasts themselves are anabolic (Figure 6 and 8).

Figure 8: Coupling of bone resorption to bone formation in healthy and disease states. In healthy adults bone formation follows bone resorption and complete replenishment of removed bone. In osteoclast-rich osteopetrosis the osteoclast number is increased, but resorption decreased, leading to accumulation of old bone (dark grey). Bone formation, however, appears to follow osteoclast number leading to increased formation of new bone (light grey). In osteoclast-poor osteopetrosis the lack of osteoclasts cause an attenuation of bone resorption, preserving old bone (dark grey). However, since no osteoclasts are present, bone formation is also affected leading to smaller and less severe bone phenotype. Adapted from (Segovia-Silvestre et al., 2008)
Evidence for Osteoclast Derived Coupling Signals

Growing evidence supports that osteoclasts themselves secrete anabolic factors not related to their resorptive function (Figure 8). The first indications that osteoclasts are anabolic came from findings in bone organ cultures showing that activation of resorption by osteoclasts led to secretion of anabolic molecules or “coupling factors” (Howard et al., 1981). Further indications that these coupling factors are secreted by the osteoclasts and not only released from degraded bone matrix were provided by studies in osteoclast-rich forms of osteopetrosis. In these forms, bone resorption is often dramatically reduced due to loss of osteoclast function, while osteoclast numbers are increased (Bollerslev et al., 1993; Del Fattore et al., 2006). Despite a lack of bone resorption, bone formation by osteoblasts is often also increased, and the number of osteoblasts have been found to correlate with the number of non-resorbing osteoclasts (Del Fattore et al., 2006). In patients with osteopetrosis due osteoclast differentiation defects, such as in RANK or RANKL deficient subjects, the bone phenotype is less severe, and bone formation is reduced compared to osteoclast-rich osteopetrotic patients (Pangrazio et al., 2012). These findings indicate that lack of osteoclasts reduce anabolic signaling leading to reduced bone formation during remodeling. In alignment with mutational studies in osteopetrotic subjects, in vivo studies in OVX rats show that inhibition of acidification inhibits bone resorption while maintaining bone formation; findings which highlight the anabolic nature of osteoclasts independent of their resorptive capability (Schaller et al., 2004). Supporting these findings are experiments showing that implantation of calcium phosphate rods containing V-ATPase inhibitors in rats attract osteoclasts but inhibit bone resorption while inducing bone formation (Rzeszut et al., 2003). PTH administration in vivo normally induces a bone anabolic response. In the osteoclast deficient c-fos -/- mouse lacking osteoclasts, administration of PTH failed to elicit an anabolic response (Demiralp et al., 2002; Luiz de Freitas et al., 2009). In contrast, c-src -/- mice which have mature but dysfunctional osteoclasts have impaired bone resorption, but maintain an anabolic response similar to wt mice in response to PTH (Koh et al., 2005), suggesting that PTH mediates its anabolic potential through the actions of osteoclasts, but independent of bone resorption. In line with animal experiments, the effect of PTH treatment is blunted in osteoporosis patients following bisphosphonate administration (Black et al., 2003). This reduction could
be caused by the ablation of osteoclasts or inhibited coupling signals following the inhibitory actions of the bisphosphonate. However, the complex nature of these mechanisms is highlighted by recent findings showing that combined Denosumab and PTH treatment leads to increased BMD in postmenopausal women (Tsai et al., 2013), suggesting that anabolic signaling by osteoclasts may be more complex than initially thought. Although data are not in complete alignment, these findings do suggest that osteoclasts secrete factors which are important for the ongoing coupling between bone resorption and bone formation during bone remodeling.

Much effort has been put into identifying coupling factors, and while findings are still controversial with no clear candidate, a number of molecules have been suggested (Pederson et al., 2008; Quint et al., 2013; Ryu et al., 2006; Baron and Rawadi, 2007; Vukicevic and Grgurevic, 2009; Ishii et al., 2009; Ishii et al., 2010; Takeshita et al., 2013; Kim et al., 2012; Dacquin et al., 2011; Negishi-Koga et al., 2011; Lotinun et al., 2013). An overview of possible candidate molecules is given in Table 3.

<table>
<thead>
<tr>
<th>Name</th>
<th>Effect on BF</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afamin</td>
<td>+</td>
<td>Pre-OB migration</td>
<td>(Kim et al., 2012)</td>
</tr>
<tr>
<td>BMP6</td>
<td>+</td>
<td>OB stimulation</td>
<td>(Pederson et al., 2008)</td>
</tr>
<tr>
<td>Cardiotrophin-1</td>
<td>+</td>
<td>OB stimulation</td>
<td>(Walker et al., 2008)</td>
</tr>
<tr>
<td>Complement component 3a</td>
<td>+</td>
<td>OB differentiation</td>
<td>(Matsuoka et al., 2014)</td>
</tr>
<tr>
<td>CTHRC1</td>
<td>+</td>
<td>OB stimulation</td>
<td>(Takeshita et al., 2013)</td>
</tr>
<tr>
<td>Semaphorin4D</td>
<td>-</td>
<td>OB inhibition</td>
<td>(Negishi-Koga et al., 2011)</td>
</tr>
<tr>
<td>Sphingosine-1-phosphate</td>
<td>+</td>
<td>OB recruitment (cell stage dependent)</td>
<td>(Pederson et al., 2008; Quint et al., 2013; Ryu et al., 2006; Lotinun et al., 2013)</td>
</tr>
<tr>
<td>TRAP</td>
<td>+</td>
<td>Osteoblast activation</td>
<td>(Del et al., 2008)</td>
</tr>
<tr>
<td>Wnt10b</td>
<td>+</td>
<td>OB stimulation</td>
<td>(Pederson et al., 2008)</td>
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Gene Therapy

Another aspect of osteopetrosis is the treatment of this disease, and while BM transplantations has proven efficient in many cases, there is a general need for alternative treatment options when lacking HLA matching donors. Much progress has been made within gene therapy in recent years and efforts are focusing on increasing efficiency and safety of this new form of therapy.

Gene therapy is based on the principle of inserting genetic information into a cell to restore or correct functionality. This approach could potentially offer a beneficial alternative to HSC transplantation when treating monogenic hematological disorders where HLA matching donors are not available.

Targeting Hematopoietic Stem Cells

A range of monogenetic diseases have by now been treated successfully with hematopoietic stem cell targeted gene therapy, such as X-linked severe combined immuno deficiency (SCID-X1) (Cavazzana-Calvo et al., 2000; Gaspar et al., 2004), adenosine deaminase SCID (ADA-SCID) (Aiuti et al., 2002) and chronic granulomatous disease (CGD) (Ott et al., 2006). Following treatment, however, several X-SCID patients developed lymphoproliferative disease due to insertional mutagenesis (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). All of these treatment approaches used gammaretroviral vectors for gene insertion indicating the need for better and safer vector design and targeting. In this context, lentiviral vectors are much less liable to insertional mutagenesis in close proximity to proto-oncogenes, increasing the general safety profile (Cattoglio et al., 2007; Neschadim et al., 2007). Not considering the serious side effects observed in SCID-X1 patients, a disadvantage of gammaretroviral vectors is also their need for cell division for transduction and integration of the pro-virus. In contrast to gammaretroviral vectors, lentiviral vectors can transduce both dividing and non-dividing cells which minimizes the need for using stimulatory cytokines to drive cell division (Naldini et al., 1996; Case et al., 1999; Miyoshi et al., 1999). Lentiviral vectors are currently being developed for gene therapy treatment in several inherited diseases, and successful gene transfer to HSCs using lentiviral vectors has been achieved in diseases such as CGD.
(Naumann et al., 2007), Wiskott-Aldrich syndrome (WASP) (Martin et al., 2005; Charrier et al., 2007), and X-linked adrenoleukodystrophy (Cartier et al., 2009).

**Lentiviral Vector Mediated Gene Transfer**

The framework of lentiviral vectors is built around the genetics of lentivirus, a member of the retrovirus family. These viruses include human immunodeficiency virus 1 (HIV-1), simian immunodeficiency virus (SIV), bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). The hallmark of retroviruses is their ability to perform reverse transcription generating DNA from two single stranded RNA copies that make up the viral genome, a process performed by the enzyme reverse transcriptase. It does so by integrating the reverse transcribed DNA into the genome of the host cell, and utilizes the cell’s machinery for transcription of viral genes (Durand and Cimarelli, 2011).

Viral vectors take advantage of the natural ability of lentiviruses to integrate into a cell and transfer genetic material. The most common lentiviral vectors are based on human immunodeficiency virus 1 (HIV-1). The severe pathology caused by wt HIV infection in humans have prompted the design of vectors where most of the viral genes have been removed, including its ability for replication. This allows for its use as delivery device for permanent incorporation of a gene of interest into a target cell genome (Schambach et al., 2013). These replication-incompetent vectors are referred to as self-inactivating (SIN) vectors. Transcription of transgenes are driven by internal promoters, allowing for careful selection of promoters depending on setting and minimizing the risk of insertional activation of neighboring genes (Miyoshi et al., 1998; Zufferey et al., 1998).

The established method for production of lentiviral vectors is by transient transfection of plasmids into a producer cell line, such as 293T. Although many of the HIV genes have been removed in today’s lentiviral production systems, reducing risk of recombination events resulting in replication competent vectors is paramount to their future use. The so-called third generation lentiviral vectors separate various elements of viral vector assembly and function onto 4 separate plasmids thereby reducing the risk of recombination (Dull et al., 1998).
Aims of the Present Study

The general aim of this thesis was to develop *in vitro* and *in vivo* models for studying osteoclasts, with special focus on the cellular aspects of the role of osteoclasts in bone remodeling and osteopetrosis.

These experiments involved the induction of osteopetrosis in adult mice by transplantation of HSCs from osteopetrotic mice. It also involved osteoclast generation from human hematopoietic progenitors and the validation of lentiviral mediated rescue of resorptive function in osteoclasts from patients with osteopetrosis.

Specific aims

1. To establish whether osteoclasts have an anabolic role *in vivo*, by developing osteoclast-rich and osteoclast-poor adult mouse models of osteopetrosis (*Papers I and II*)

2. To investigate whether osteoclasts, independent of bone resorption, secrete anabolic signals for osteoblasts *in vitro* (*Paper III*)

3. To characterize the correction of resorptive function in osteopetrosis patient CD34+ cells using a lentiviral based gene therapy approach to rescue TCIRG1 function *in vitro* (*Paper IV*)
Summary of Findings

Paper I

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, 2011; 6(11): e27482*

Bone resorption by osteoclasts and bone formation by osteoblasts are under normal circumstances tightly balanced, a process referred to as coupling. Studies within the last decade suggest that the coupling mechanism is more complex than initially thought and that osteoclasts play a greater part in regulation via secreted anabolic factors, independent of bone resorption capability.

Patients and mice with mutations reducing the ability of the osteoclasts to secrete acid have osteopetrosis, characterized by defective bone resorption, increased osteoclast numbers, and, interestingly, normal or even increased bone formation. However, the developmental nature of these phenotypes, particularly the large accumulation of calcified cartilage in the bones and the short life span, limits the general applicability of these findings. To overcome this we used fetal liver cell (FLC) transplantation from *oc/oc* mice with non-resorbing osteoclasts to replace the BM of irradiated fully grown adult mice and, hereby, inducing an osteopetrotic phenotype. We then observed the effects on bone volume and particularly strength over a 3 to 6 months period.

In summary, we found that *oc/oc* transplanted mice had significantly reduced bone resorption, while the osteoclast numbers were increased compared to control transplanted animals. Bone formation was elevated at week 6 after transplantation but normalized after 12 weeks and reduced from here on. μCT analysis showed a significant increase in both femoral and vertebral bone volume. These increases manifested in increased bone strength in both compartments. The increases were apparent already after 3 months in the vertebrae, while femoral changes took longer, probably due to differences in remodeling rate between the two compartments. In conclusion, these data suggests that attenuation of acid secretion by osteoclasts in adult mice leads to an unbalancing phenotype and increased bone strength; findings which are important for future development of non-catabolic therapies in bone metabolic diseases.
A comparison of osteoclast-rich and osteoclast-poor osteopetrosis in adult mice sheds light on the role of the osteoclast in coupling bone resorption and bone formation.

Calcified Tissue International, 2014; 95(1):83-93

After having established that transplantation of oc/oc FLCs could induce a mild unbalancing phenotype where bone formation was maintained despite inhibition of bone resorption, we wanted to further investigate the role of osteoclasts in controlling bone remodeling by comparing our current osteoclast-rich model to an osteoclast-poor system with no or very few osteoclasts. Current osteoporosis treatments largely seek to inhibit the osteoclasts and in doing so generally eliminate them. The great drawback to these anti-resorptive treatments is the secondary loss of bone formation happening from the coupling mechanism between osteoblasts and osteoclasts. By inhibiting osteoclastic acid secretion instead of abolishing osteoclasts, you possibly maintain bone formation, thus increasing the overall efficacy on turnover.

In this study we investigated the differences between osteoclast-rich oc/oc and osteoclast-poor RANK KO transplanted mice and two corresponding control groups, and analyzed changes in osteoclast profiles, bone volume, bone strength and bone formation parameters. Transplantation of FLCs resulted in a mean engraftment ratio of approximately 98%. TRAP5b, a marker of osteoclast numbers, and osteoclast counts were 50% increased in the oc/oc recipient group as expected from previous studies (paper I). Osteoclast numbers were decreased in RANK KO recipients, but not completely abolished, despite 98% engraftment ratio. Analysis of in vitro differentiated splenocyte derived osteoclasts from oc/oc recipients showed a high number of non-resorbing osteoclasts, while splenocytes from RANK KO recipients failed to develop into bone resorbing osteoclasts. Serum CTX-I was equally decreased in both oc/oc and RANK KO recipients compared to respective controls. Bone volume and bone strength were equally increased in the femur, while in the vertebrae changes in oc/oc recipients were higher than for RANK KO recipients compared with respective controls. Finally, dynamic histomorphometry showed an increase in MAR as well as bone formation rate per bone surface in both oc/oc and RANK KO recipients compared to controls. However, changes were more prominent in the oc/oc recipients. In conclusion, these data indicate that unbalancing of bone remodeling via oc/oc and RANK KO FLC transplantation into adult wild type (wt) mice leads to equal declines in bone resorption, but increased bone formation when osteoclast are preserved in vivo. These findings rationalize targeting the acid secretion process when developing treatments for bone metabolic diseases.
A specific subtype of osteoclasts secretes factors inducing nodule formation by osteoblasts.

*Bone, 2012; 51(3):353-61*

Osteoclasts are important for the coupling process between bone resorption and bone formation during bone remodeling. The aim of this study was to investigate at what stage osteoclasts are anabolically active and whether matrix or resorptive function affects this signaling.

Human monocytes were differentiated into osteoclasts driven by the presence of M-CSF and RANKL. Conditioned media (CM) was collected from cultures of macrophages, pre-osteoclasts or mature functional or osteopetrotic osteoclasts. The cells were either differentiated on plastic, bone or dentin and with or without the inhibitors diphyllin, GM6001, or E64. The osteoblastic cell line 2T3 was then treated with conditioned media or non-conditioned control media for 12 days and bone formation analyzed with Alizarin Red.

Osteoblasts incubated with CM from mature osteoclasts induced bone formation while conditioned media from macrophages did not. Media from non-resorbing ARO osteoclasts still induced bone formation by osteoclasts despite significantly lowered bone resorptive activity. In alignment, conditioned media from osteoclasts treated with the V-ATPase inhibitor diphyllin or the cysteine protease also induced bone formation, although the effect was slightly reduced, compared to vehicle treated osteoclasts, while conditioned media from osteoclasts treated with MMP-inhibitor GM6001 were comparable to vehicle conditioned media. Finally, osteoclasts seeded on either decalcified bone or dentine generated conditioned media that had strongly impaired anabolic output.

The data suggest that osteoclasts, dependent and independent of their resorptive capabilities can secrete anabolic signals which induce bone formation. Importantly the data also show that the anabolic capability is very much dependent on the type of matrix that cells are present on, since both dentine and decalcified bone impaired the anabolic response. These findings are in line with studies in patients with osteopetrosis, where mutations in the osteoclast resorption machinery abolish resorptive function but maintain osteoclast viability, and importantly bone formation.
Lentiviral gene transfer of TCIRG1 into peripheral blood CD34(+) cells restores osteoclast function in infantile malignant osteopetrosis.


ARO (IMO) is a rare genetic disease, and is lethal in infancy in the absence of matching BM donors. The aim of this study was to rescue the resorptive function of osteoclasts derived from ARO patients using lentiviral mediated gene transfer of TCIRG1 cDNA to osteoclast precursor cells. CD34+ progenitor cells were isolated from peripheral blood samples from 5 IMO patients and from cord blood samples of healthy controls. They were then transduced with a self-inactivating (SIN) lentiviral vector containing TCIRG1 cDNA and a green fluorescent protein (GFP) marker gene. The cells were expanded (500-fold) for 2 weeks in the presence of myeloid acting cytokines, and gradually changed from CD34 expressing to CD14 expressing cells. Following the expansion, cells were seeded on bovine bone slices and differentiated into mature osteoclasts for 10 days, in the presence of M-CSF and RANKL, and osteoclastogenesis, osteoclast activity, and expression of TCIRG1 were investigated.

The transduction efficiency after 2 weeks of proliferation reached approximately 40%. During maturation ARO CD34+ cells as well as transduced cells developed into large multinucleated osteoclasts in a similar fashion to cord blood CD34+ cells, expressing the osteoclast marker TRAP. The osteoclasts also maintained a similar morphology visualized by the presence of the characteristic actin ring. However, ARO osteoclasts completely failed to resorb bone, as shown by a decreased calcium secretion and CTX-I release. Transduction of CD34+ IMO cells resulted in increased TCIRG1 mRNA and protein levels, as shown by qPCR and western blot; an expression which was comparable to controls. However, TCIRG1 protein was only present at the late stages of maturation similarly to wt osteoclasts. Vector corrected ARO osteoclasts generated increased Ca²⁺ and CTX-I into the media and formed clearly visible resorption pits. The average resorption was approximately 70 to 80% of that of osteoclasts generated from normal CD34+ cord blood cells. Furthermore, transduced CD34+ cells engrafted successfully in NSG-mice, and no signs of toxicity were observed. Finally osteoclasts generated from isolated splenocytes expressed GFP in vitro, confirming engraftment.

Overall, these findings justify further development of gene therapy targeting osteoclasts in the treatment of osteopetrosis.
General Discussion

Diseases affecting osteoclast development and function as well as cell culture models of osteoclasts have been invaluable tools in the understanding of osteoclast regulation and function, and have greatly assisted in the development of treatments for diseases caused by osteoclast dysfunction.

The work in this thesis has been divided into two parts; 1) a series of in vitro and in vivo studies investigating the role of osteoclasts in regulating bone remodeling and, more specifically, bone formation. The rationale for this line of work is based on the hypothesis that osteoclasts independent of their resorptive capability secrete anabolic factors important for ongoing bone formation, as indicated in patients with osteoclast-rich osteopetrosis. Hence, abolishing resorption through inhibition of acidification, and maintaining the presence of non-resorbing osteoclasts might be an advantageous mode of action for treating bone turnover diseases, compared to current available therapies. 2) An in vitro based study developing an osteoclast model for validation and development of correction of osteopetrosis in patient cells. The long term aim of this line of experiments is to develop hematopoietic stem cell targeted gene therapy for ARO patients with mutations in TCIRG1.

Investigating Osteopetrosis in Adult Mice

The work in paper I and II has largely focused on the development of an in vivo model that could be used to investigate the mode of action by which bone formation is maintained in osteopetrotic subjects, independent of bone resorption but not osteoclast presence. The obvious comparison would be a model which has no osteoclasts, and, hence, potentially a lowered bone formation as an effect of this, such as in osteoclast poor osteopetrosis (Guerrini et al., 2008; Pangrazio et al., 2012).

The induction of osteopetrosis was performed in adult mice for several reasons. The endogenous mouse models of ARO, the oc/oc and the RANK KO mouse, suffer from severe developmental phenotypes in their bones. The lack of bone resorption due to lack of acidification or lack of osteoclasts in general, results in the inability to dissolve the inorganic part of the calcified cartilage which is used to mold the bones during bone growth (Dougall et al., 1999; Neutzsky-Wulff et al., 2010). This leads to the development of bones which largely consist of calcified cartilage instead of normal
bone and renders them weak and brittle. The undegraded calcified cartilage occupies marrow space, effectively blocking the cavity for proper hematopoiesis (Dougall et al., 1999; Neutzsky-Wulff et al., 2010). These traits of the endogenous models lead to early death, limiting their applicability in the investigation of adult mice. To overcome this, we induced osteopetrosis in adult mice by transplanting FLCs from osteopetrotic mice. Using an adult chimera model allowed us to properly investigate the unbalancing phenotype between osteoclasts and osteoblasts independent of the developmental phenotype seen in endogenous models, and to isolate remodeling mechanisms from early modeling and endochondral ossification processes related to growth. In addition, a transplantation model gave us the opportunity to observe the effect of inhibiting acidification in old mice instead of young. A treatment targeting acidification would mainly be developed for the treatment of osteoporosis, a disease primarily manifesting in the older population. Hence, from a translational perspective using adult mice is favorable compared to using actively growing mice for these purposes.

Phenotype Penetrance in Adult Osteopetrotic Mice

As discussed in paper I and II, we were surprised by the mild phenotype in the transplantation models. While realizing that there are differences in terms of magnitude of phenotype in the oc/oc model described in the two papers, we speculate that the fairly mild phenotype in the experiment in paper II is the explanation for the lack of significant increase in vertebral bone volume in the RANK KO, an effect which was expected. In terms of why the bone histomorphometry in paper I had a different outcome, this might be related to the differences observed in phenotype. In paper I it is clearly seen that a peak bone volume effect is obtained in vertebrae already after 12 weeks, and this is unchanged at 28 weeks. These data indicate that coupling has been reestablished in these animals, which fits with the biomarker and histomorphometry data. In paper II we observed a smaller increase at 15 weeks, which is best explained by the fact that the phenotype is still accumulating, and, while very speculative, we might see corresponding increases in bone formation rates in the previous studies at an earlier time point, before a plateau is reached. We do not feel that the time frame differences between these two experiments cause the differences, but rather the milder manifestation of the phenotype; although, this cannot be excluded.

Engraftment levels reached 95-98% leaving a minor percentage of host cells still remaining in recipient animals. It is possible that HSCs in this remaining pool can divide and give rise to a number of functional osteoclasts and, thereby, limiting the effect of the osteopetrosis induced in these studies.
Another possible explanation for the mild phenotype can be the existence of quiescent osteoclast precursors (QOPs). QOPs are believed to be post mitotic osteoclast precursors, residing both in circulation and on bone surfaces in proximity to osteoblasts (Mizoguchi et al., 2009; Muto et al., 2011). This specific pool of precursors expresses high levels of RANK and c-fms and supposedly exists in a quiescent state and has a longer half-life than normal osteoclasts (4 weeks longer). The post mitotic characteristic is a key feature of QOPs, and renders this population resistant to 5-FU, a strong inducer of apoptosis in cells with high proliferative potential, as well as the inhibitor of DNA replication hydroxyurea (Mizoguchi et al., 2009). These findings, while not experimentally confirmed, also imply resistance of QOPs to conditioning regimens targeting high-proliferation cells such as radiation conditioning. In papers I and II we use lethal radiation doses to ablate the BM including any proliferating myeloid precursors to condition mice for stem cell transplantation. Despite this conditioning we still observe osteoclasts in recipient mice, a finding not related to the RANK KO model itself, as the endogenous model as well as patients are completely free of osteoclasts (Guerrini et al., 2008; Pangrazio et al., 2012; Dougall et al., 1999).

Thus it is possible that QOPs remain in vivo in circulation or on bone surfaces, and can be recruited to sites of bone remodeling, even after radiation therapy. This would account for the amount of osteoclasts in RANK KO recipients, and possibly explain the fairly mild phenotype observed. Whether oc/oc recipients have the same amount of QOPs would require further analysis, such as analysis of donor and host specific surface proteins in the osteoclast population. A way to remove all osteoclasts in a transplantation model might be to transplant stem cells from mice with inducible expressing of diphtheria toxin driven by the cathepsin K promoter (ctskCre;DTA), as recently published by Oury and colleagues (Oury et al., 2013). The fusion of engrafted osteoclast precursors and QOPs during osteoclastogenesis lead to entry of diphtheria toxin in the QOPs, causing a complete ablation of mature osteoclasts. Diphteria toxin driven by the cathepsin K promoter might solve the issue with osteoclast generation from QOPs, as the mechanism affects maturating osteoclasts and not proliferating cells. However, this would of course not be applicable in oc/oc recipients, as we require the osteoclast to be present in this model, overall limiting the potential of the diphtheria toxin model in a comparative study.

The ideal adult models of osteoclast-rich and osteoclast-poor osteopetrosis for studying how osteoclasts affect bone remodeling and particularly bone formation in vivo would be to generate inducible knock-out models targeting the genes encoding TCIRG1 and RANK.
Secretion of Coupling Factors is Dependent on Osteoclast Subtype

The *in vivo* studies clearly support the hypothesis that non-resorbing osteoclasts are anabolic; however, the understanding of when and how they produce these factors is limited. Therefore, in paper III, we undertook an extensive analysis of the anabolic signals as a function of cell phenotype and matrix composition.

CM from mature human osteoclasts grown on bone and plastic induced nodule formation when added to osteoblast cultures. In contrast, CM from neither pre-osteoclasts nor macrophages grown on bone were able to induce this nodule formation by osteoblasts. This is in line with previous findings showing that osteoclast precursors are not sources of anabolic signals (Pederson et al., 2008), as well as findings of reduced bone formation in osteoclast-poor osteopetrosis patients (Pangrazio et al., 2012).

An anabolic signal have been speculated to be a mix of both osteoclast derived factors as well as signals released from the matrix during bone resorption as has been extensively reviewed (Henriksen et al., 2009b; Henriksen et al., 2011; Henriksen et al., 2014; Karsdal et al., 2007; Martin et al., 2009; Martin, 1993; Martin and Sims, 2005; Martin and Seeman, 2008; Sims and Walsh, 2012; Sims and Vrahnas, 2014).

We found that both the acidification inhibitor diphyllin and the cathepsin K inhibitor E64 inhibited the release of anabolic factors when grown on bone; an effect which was absent in osteoclasts grown on plastic. These findings suggest that anabolic factors are also released from the degraded bone and that this release can be inhibited using inhibitors of bone resorption. In further support of this was the observation that acidification deficient ARO osteoclasts showed similar blunting of anabolic signaling when grown on cortical bone. These findings strengthen the idea that an anabolic signal from osteoclasts during bone remodeling is a mix of molecules released from the resorbed matrix in addition to factors released from mature osteoclasts independent of resorption.

A peculiar finding in this study was the complete absence of anabolic signaling when osteoclasts were grown on alternative substrates such as dentine or decalcified matrix. Dentine is a highly calcified matrix which under normal conditions is non-remodeled. It is likely that dentine contains inhibiting signals which limit the release of anabolic signals by osteoclasts, thus abrogating bone remodeling. In line with this, osteoclasts grown on decalcified matrix showed a similar lack of anabolic signals, again indicating an active role for matrix type. The effect of matrix composition, while speculative, could indicate that various osteoclast subtypes have different resorption function and anabolic action directly induced by signals on matrix level.
Level of Correction for Gene Therapy of ARO

For future development of gene therapy treatment of osteopetrosis an important question is what level of osteoclast function is needed for correction of disease phenotype.

Recent mouse experiments have indicated that only minor reconstitution (5 to 10%) of the BM is necessary to incur rescue of resorptive function in vivo (Flores et al., 2010). In line with this, human studies in individuals with mutations in only one allele of TCIRG1 show no signs of osteopetrosis indicating haplosufficiency.

The low number of rescued cells needed for functional correction might be explained by the fusing nature of osteoclasts formation. It is possible that wt or corrected monocytes can fuse with, and correct, dysfunctional monocytes during osteoclast differentiation, resulting in an overall higher number of rescued osteoclasts (Figure 9). In alignment, transducing CD34+ cord blood with a GFP containing vector gave a 30% transduction efficiency; an efficiency which after differentiation resulted in GFP expression in almost 100% of mature osteoclasts.

To see how the fusion mechanism relates to function, we setup a series of experiments where CD34+ ARO cells, TCIRG1 transduced ARO CD34+ cells, and cord blood CD34+ cells were mixed at different ratios, differentiated into osteoclasts on bovine bone slices and analyzed for bone resorption capacity. Findings indicate that small amounts of cord blood CD34+ cells are enough to restore resorptive function in ARO co-culture. These data are in line with the GFP expression pattern seen in fusing GFP transduced osteoclasts, in vivo rescue transplantations, as well as the haplo sufficient nature of ARO related to TCIRG1 mutations. The increase in function does not appear to be caused by selective expansion or differentiation of cord blood cells, as we saw similar results when mixing ARO CD34+ cells with TCIRG1 transduced ARO CD 34+ cells. Together, these findings indicate that even low transduction and engraftment efficiencies might be sufficient for functional reconstitution of osteoclasts when treating ARO.

An interesting parameter to determine would be the maximum rescue achievable in vitro; however, transduction of CD34+ cells with current vectors rarely exceeds 30% transduction efficiency, which limits the “dose range”. Sorting cells using fluorescence activated cell sorting (FACS) might be possible to enrich the number of transduced cells, but pilot experiments indicate that cell viability is heavily affected, limiting the use of the method in this setup.
Figure 9. Osteoclast fusion might increase rescue percentage
Schematic figure of how osteoclast fusion might impact overall rescue percentage in mature osteoclasts. If we assume that osteoclasts fuse randomly, transduced and non-transduced pre-osteoclasts will fuse in ratios correlating to the transduction efficiency, hereby increasing the overall amount of cells with a corrected TCIRG1 gene.

Post-transcriptional Regulation of Vector Driven TCIRG1 Expression

Osteoclastogenesis is a tightly regulated process which is highly dependent on the osteoclastogenic cytokines M-CSF and RANKL. The differentiation into osteoclasts involves the up-regulation of a number of osteoclast specific genes and proteins, the most prominent being cathepsin K, ClC-7, MMP9, TRAP, and TCIRG1.

It is well-established in the literature that TCIRG1 is regulated at the transcriptional level by a number of transcription factors. In pre-osteoclasts, the TCIRG1 gene is repressed by the poly (ADP-ribose) polymerase-1 (PARP-1)(Beranger et al., 2006). During osteoclastogenesis, the presence of RANKL causes degradation of PARP-1 and upregulation of TCIRG1 through JunD proto-oncogene (junD) and Fos related antigen (Fra-2)(Beranger et al., 2007).

Driving a transgene by strong viral promoter such as the spleen focus forming virus (SFFV) promoter most often causes it to be expressed ectopically, in cell lineages and at differentiation stages not usually relevant to the normal endogenous gene. In paper IV the expression of TCIRG1 protein was therefore expected to be present at both
the pre-osteoclast stages as well as in mature osteoclasts, when cells were transduced with a TCIRG1-containing vector. Counter intuitively, we observed no TCIRG protein in pre-osteoclasts. TCIRG1 protein became detectable at day 5 to 7 of osteoclast maturation, resembling the endogenous transcriptional regulation previously described. Co-expressed GFP protein as well as exogenous TCIRG1 mRNA was present in pre-osteoclasts and throughout differentiation, consistent with exogenous expression. Furthermore, transduced cells cultured without RANKL differentiated into macrophages but failed to express TCIRG1 protein indicating that the expression is mediated through RANKL or osteoclast maturation. These observations indicate that the expression of TCIRG1, in addition to transcriptional control, is regulated through a post-transcriptional mechanism. Interestingly, HT1080 cells transduced with the TCIRG1-vector expressed TCIRG1 protein early on, indicating that the mechanism was absent in non-hematopoietic cells. The mechanism of this post-transcriptional regulation is unclear, but could involve proteasomal degradation caused by regulated intracellular processes such as folding or compartment sorting, or regulation through RNA binding proteins ensuring expression at the correct cell stage (Glisovic et al., 2008).

If vector mediated expression of TCIRG1 is regulated post-transcriptionally in a manner resembling the endogenous regulation, this might in the gene therapy setting eliminate the need for cell specific promotors in the vectors, since TCIRG1 protein would be expressed only in mature osteoclasts.
Future Perspectives

Identifying Osteoclast Derived Anabolic Factors

Future experiments will aim at identifying factors expressed by the osteoclasts which direct the anabolic actions of the osteoblasts. We showed in paper III, that the anabolic actions of osteoclasts affecting the nodule formation by osteoblasts are highly dependent on osteoclasts stage and matrix composition. The secretion of anabolic factors from osteoclasts grown on cortical bone, and the absence of these factors on dentine, as well as in macrophages provides a unique opportunity to investigate differences in expression patterns. Briefly, RNA and protein from osteoclasts grown on matrices differentially affecting their anabolic potential will be isolated, and gene and protein expression analyzed using affymetrix array and proteomic analysis. Once a gene or molecule is identified, the role of such a molecule can investigated and possibly validated by constructing gene specific knockout mice, as was recently done for the potential coupling factor Cthrc1 (Kimura et al., 2008; Takeshita et al., 2013), or combining lentiviral knockdown of potential factors with the adult osteopetrotic mouse model. The identification of a coupling factor holds great potential in both therapeutic development and biomarker assessment of bone related diseases.

Targeting Acidification When Treating Bone Turnover Diseases

Mutations in osteopetrotic patients and animal models with acidification deficiency have highlighted the importance of having osteoclasts present to maintain ongoing bone formation (Bollerslev et al., 1989; Bollerslev et al., 1993; Frattini et al., 2000; Henriksen et al., 2004; Henriksen et al., 2009a; Kornak et al., 2000; Kornak et al., 2001; Segovia-Silvestre et al., 2008). Most importantly, the finding that osteopetrotic subjects, despite having a high number of non-resorbing osteoclasts, maintain normal or even increased bone formation, suggest that osteoclasts, but not necessarily their resorptive function, are a driving factor in maintaining bone formation locally at sites
where bone formation is needed (Henriksen et al., 2004; Henriksen et al., 2014; Karsdal et al., 2007; Sims and Walsh, 2012).

These findings clearly distinguish inhibition of acid secretion through inhibition of for example ClC-7 or the osteoclastic V-ATPase in terms of treatment mode of action in bone turnover diseases, from traditional anti-resorptives such as bisphosphonates or the recently approved RANKL antibody, Denosumab (Figure 10). These current anti-resorptive treatments, despite being very potent inhibitors of bone resorption, suffer from secondary reduction in bone formation. This reduction in bone formation limits long-term efficacy, and at the same time it is not completely clear how this “freeze” of the remodeling process affects general bone health.

Currently the only approved anabolic treatment is PTH or fragments hereof. However, secondary to the anabolic effect of PTH, are catabolic increases in osteoclast mediated bone resorption limiting the overall efficacy of the drug. To overcome this limitation, combinational treatments with PTH and bisphosphonates seeking to limit the secondary catabolic response from the osteoclasts have been tested in clinical trials, but data indicate a blunting of the anabolic response, which have been proposed to be mediated by a reduction in osteoclast numbers (Black et al., 2003). However, recent studies showing osteogenic effects of PTH in combination with Denosumab question these findings and suggest that mode of action of PTH might be more complex (Tsai et al., 2013).

Several molecules with proposed superior modes of action are currently in clinical development. The cathepsin K inhibitor Odanacatib inhibits collagenolysis while maintaining osteoclasts, and is thus proposed to maintain ongoing osteoblastic bone formation (Gauthier et al., 2008). However, thus far clinical trials have failed to provide compelling evidence that bone formation is ongoing in face of reduced bone resorption, although the reduction in biomarkers of formation is less than seen for bisphosphonates and Denosumab (Bone et al., 2010; Eisman et al., 2011). The reason for the observed decrease in bone formation is currently unclear, but the mechanism might involve modified signaling at osteoclast attachment sites (Henriksen et al., 2009b; Karsdal et al., 2007).

Where Odanacatib is proposed to work through mechanisms of maintaining bone formation similar to inhibition of acid secretion, the antibody anti-sclerostin exerts its effect through the osteoblastic lineage. Its mode of action is mediated through the binding of sclerostin; an osteocyte secreted inhibitor of the Wnt/beta-cathenin pathway (Poole et al., 2005). This binding results in osteoblast activation leading to bone formation (Li et al., 2009; McClung et al., 2014). It should be noted that despite its anabolic profile, anti-sclerostin also decreases bone resorption, but the reason for this is currently not understood (McClung et al., 2014). The effect of not having the osteoclasts directly involved in guiding the bone forming actions of the osteoblasts as seen in normal bone remodeling have not yet been fully investigated in long term clinical trials. It is possible that bone formation is instead activated in a
systemic manner similar to bone modeling; a differing mode of action which could potentially lead to increases in bone at unwanted sites such as joints or nerve endings.

All in all, although bone turnover diseases are well treated, there is still room for new treatments. Acidification inhibitors, acting locally on osteoclasts, and maintaining bone remodeling in the BRU including bone formation, similar to the normal physiological state, could be superior to current modalities.

![Graph showing treatment modalities for osteoporosis](image)

**Figure 10. Treatment modalities for osteoporosis**
Schematic representations of the correlation between osteoclast number, bone resorption and bone formation in different treatments. BP, bisphosphonate. Adapted from (Karsdal et al., 2007)

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**Critical Issues for Further Development of Clinical Gene Therapy**

**Safety concerns**

When developing gene therapy history calls for careful consideration of the safety of treatment. Clinical gene therapy trials targeting hematopoietic stem cells have now been successfully performed in a range of diseases, including ADA-SCID, SCID-X1 and Wiskott Aldrich syndrome (Braun et al., 2014). However, a number of clinical gene therapy trials have resulted in serious side effects due to genotoxicity, in many cases leading to leukemia. Among these are chronic granulomatous disease, SCID-X1, and Wiskott-Aldrich (Stein et al., 2010; Braun et al., 2014; Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). These trials have generally made use of gamma-retroviruses for viral transduction, and the link between vector origin and insertional mutagenesis has naturally led to a great effort being invested in developing safer gene delivery methods (Naldini et al., 1996; Case et al., 1999; Miyoshi et al.,}
one of the primary objectives of this thesis has been to develop and validate a system for transduction of osteoclast precursors to be used in the development of gene therapy for osteopetrosis. After having shown that osteoclast function can be corrected through rescue of the TCIRG1 gene in CD34+ cells from osteopetrotic patients we are now aiming at gene therapy development for clinical application. In this regard, safety is a prime aspect and the SFFV promoter, used up to this point as a proof of concept vector, has to be exchanged with cellular promoters derived from human genes. We are currently testing vectors making use of the mammalian promoters elongation factor 1 alpha (EF1-alpha) or ChimP. Preliminary data indicate that transduction efficiencies and correction are lower than SFFV-promoter driven TCIRG1 expression \textit{in vitro}, but sufficient for functional rescue. Further studies will be aimed at optimizing transduction protocols to be in line with further clinical development.

\textit{In vivo models of correction using human CD34+ cells}

It is clear that clinical development of gene therapy not only requires preclinical optimization of therapeutic vectors but also the use of relevant mouse models. Efforts are currently put into the development and optimization of a xenograft model, which can be used to study disease correcting treatment strategies of human patient cells in \textit{vivo}. The immunodeficient NSG mouse has proved valuable in analyzing the transplanted cells ability to graft, and assessment of vector mediated toxicity. The NSG mouse is, however, limited in terms of studying functional rescue due to lack of cross reactive cytokines, especially M-CSF, which causes lack of human myeloid differentiation \textit{in vivo}. Therefore, it might be necessary to isolate the CD34+ population from transplanted NSG mice, which can then be differentiated \textit{ex vivo} and analyzed for osteoclastogenic potential after \textit{in vivo} transplantation. Finally, the NSG mouse is not an osteopetrotic model and, as such, is not suitable for functional analysis of rescue in vivo. Such an analysis may require a combination of the immunodeficient properties of the NSG mouse, with the osteopetrotic features of the oc/oc model.
Selection of patients and clinical treatment scenario

Clinically, a gene therapy trial will be offered and aimed at children diagnosed with ARO, which are without a suitable stem cell donor. Due to the high prevalence of CD34+ cells in peripheral blood of IMO patients, cells will be harvested through exchange transfusions without the use of mobilization regimens (Steward, Blair et al 2005). CD34+ cells will be transduced with the optimal therapeutic vector based on preclinical validation. Depending on the need for multiple exchange transfusions, transduced cells will be either kept in culture or frozen before being re-infused into the patient. It is unclear whether myeloablation will be needed for stable engraftment of cells, but in order to ensure adequate space in the niche, patients will be treated with low-dose busulphan, similar to ADA-Scid and CGD trials, as well as the oc/oc model (Askmyr et al., 2009b; Aiuti et al., 2002; Ott et al., 2006). Follow up will be performed regularly analyzing the patients for clonal composition of hematopoiesis (Schmidt et al., 2007).

Figure 11. Schematic outline of proposed clinical gene therapy setup.
Popular Scientific Summary

My PhD thesis has focused on increasing the understanding of the osteoclasts, the cells responsible for degradation of bone, by studying the rare hereditary disease autosomal recessive osteopetrosis (ARO). ARO is present already at birth and is caused by mutations affecting the osteoclasts ability to degrade bone. Non-functional osteoclasts lead to dramatic increases in bone thickness affecting bone marrow, blood vessels, and nerves due to having smaller cavities in the skeleton. Despite the increase in bone mass, the bones are weak and brittle because of how the bones are molded during growth, and fractures often occur. In almost half the cases, the disease is caused by mutations in the gene called TCIRG1, which encodes a protein important in the degradation of calcium in bone. As osteoclasts come from blood stem cells, the disease can be corrected by bone marrow transplantation, replacing sick stem cells with healthy cells from a donor. However, this treatment is risky and is highly dependent on a matching donor. Without bone marrow transplantation the disease is fatal within five years of birth.

Under normal circumstances the osteoclast mediated bone degradation process leads to release of signals that direct new bone formation by specialized cells called osteoblasts. In ARO patients these osteoclast derived signals are maintained despite the lack of bone degradation, suggesting that simply the osteoclasts being present may be enough for bone formation. A great effort has been put into identifying these osteoclast derived signals as they may lead to new treatments for patients with too little bone, such as osteoporosis.

In the first two papers we developed an adult mouse model with osteopetrosis to investigate how signals from non-functional osteoclasts affected bone formation. We induced osteopetrosis by transplanting stem cells from osteopetrotic mice, with defects in the mouse version of TCIRG1, into normal adult mice. The healthy osteoclasts are thereby replaced by non-functional osteoclasts and the mice develop a mild form of osteopetrosis. In paper I we found that these “osteoclast-rich” osteopetrotic mice have increased bone mass and surprisingly increased bone strength compared to control mice.

In Paper II we compared the osteoclast-rich model to an osteoclast-poor model. For this we instead used stem cells from a mouse model which has a mutational defect in a gene crucial for osteoclast development. Comparing these two types of osteopetrosis in mice, we found that bone formation was increased in osteoclast-rich osteopetrotic
mice compared to their osteoclast-poor counterparts. These findings suggest that blocking bone degradation, while keeping osteoclasts, maintains the signals directing bone formation. A molecule blocking the osteoclasts ability to degrade bone but without removing the cell itself, may be interesting as potential treatment for low bone mass diseases such as osteoporosis.

In paper III we used different ways of modifying osteoclast function to investigate how the release of signals from osteoclasts, directing bone formation is affected by these changes in various osteoclast related functions. We also used osteoclasts from ARO patients to show that signals are still being released directing bone formation despite their lack of bone degradation. The studies indicate that the signals are both dependent on how old the osteoclast is, and what type of bone the osteoclast is degrading.

Blood stem cells can renew themselves and produce all the different kinds of blood cells in the blood throughout a lifetime. By correcting these cells one can change the function of all osteoclasts that are to be made. The principle of gene therapy is to insert the normal functional gene into a cell having dysfunctional gene. This may result in the patient’s ability to produce the correct functional protein themselves.

In Paper IV we used a modified HIV-virus as a tool to insert the correct functioning TCIRG1 gene into human ARO blood stem cells. We found that the corrected cells expressed the functional protein and that function was almost fully restored in these osteoclasts, as could be measured by the release of degradation products such as calcium and collagen fragments.

In summary these studies have increased the understanding of ARO and the osteoclast related defects causing this disease as well as taken a step towards gene therapy as a treatment for this ARO. The experiments have also shed light on the important regulation undertaken by the osteoclasts in the maintenance of bone, and findings suggest that targeting the osteoclasts ability to degrade bone without removing the osteoclasts may be a novel target for treatment of bone turnover diseases.
Articles not Included in this Thesis

Disruption of the V-ATPase functionality as a way to uncouple bone formation and resorption – a novel target for treatment of osteoporosis


*Curr Protein Pept Science, 2013; 13(2):141-51; (Review)*

Osteoclasts are not crucial for hematopoietic stem cell maintenance in adult mice


*Haematologica, 2013; 98(12):1848-55*
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