The Role of Sorting Nexin 10 (Snx10) in Control of Osteoclast Function and Regulation of Bone Homeostasis

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The role of sorting nexin 10 (Snx10) in control of osteoclast function and regulation of bone homeostasis

A dissertation presented

by

Liang Ye

to

the Department of Biological Sciences in Dental Medicine

in partial fulfillment of the requirements

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in the subject of

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The role of sorting nexin 10 (Snx10) in control of osteoclast function and regulation of bone homeostasis

Abstract

Sorting Nexin 10 (Snx10) is expressed in osteoclasts and is required for osteoclastic bone resorption \textit{in vitro}. To study the role of Snx10 in osteoclastic bone resorption and bone homeostasis \textit{in vivo}, we investigated the expression of Snx10 and created mouse models in which Snx10 was deficient in osteoclasts or globally. Osteoclast-specific Snx10-deficient mice exhibited severe osteopetrosis with abnormal bone micro-architectural parameters \textit{in vivo}, consistent with the failure of osteoclasts to normally resorb bone. Osteoclast-derived Snx10 deficiency didn’t completely inhibit osteoclast formation, however, the capacity to resorb bone was significantly reduced. Intracellular vesicular transport, ruffled border formation and extracellular acidification were found to be severely impaired due to osteoclast-derived Snx10 deficiency. We also discovered that Snx10 was highly expressed in gastric zymogenic cells, with mutations leading to gastric dysfunction and low calcium solubilization. Global Snx10-deficiency in mice results in a combined phenotype: osteopetrosis (due to osteoclast defect) and rickets (due to gastric dysfunction and low calcium availability, resulting in impaired bone mineralization and hypocalcemia). Osteopetrorickets, the paradoxical association of insufficient mineralization in the context of a positive total body calcium balance, was thought to occur due to failure of the osteoclasts to maintain normal
calcium homeostasis. However, osteoclast-specific Snx10 deficiency had no effect on calcium balance, and therefore led to severe osteopetrosis without rickets. Moreover, supplementation with calcium gluconate prevented the rachitic phenotype and rescued the early death in global Snx10-deficient mice, suggesting that this may be a life-saving component of the clinical approach to Snx10-dependent human ARO with hypocalcaemia and/or no improvement after HSCT. We concluded that tissue-specific effects of Snx10 mutation need to be considered in clinical approaches to this disease entity. Reliance solely on hematopoietic stem cell transplantation can leave hypocalcemia uncorrected with sometimes-fatal consequences.

To our knowledge, this is the first study to explore the role of Snx10 using the genetically modified mouse model. This study not only uncovered the cellular mechanism by which Snx10 regulates osteoclastic bone resorption but also established an essential role for Snx10 in bone homeostasis and underscore the importance of Snx10-dependent gastric function in calcium homeostasis.
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CHAPTER 1

General Introduction
General Introduction

Bone resorption by osteoclasts

Bone resorption by osteoclasts is essential for normal skeleton development and physiology. The pathophysiology of several skeletal diseases is associated with either increased (osteoporosis, metastatic bone disease and Paget's disease) or decreased (various types of osteopetrosis, osteopetrorickets and pycnodysostosis) bone resorption (Russell et al., 2001). Bone resorption by osteoclast is a lifelong process regulated by a series of cytokines and hormones such as Interleukin 1 (IL-1), interleukin 6 (IL-6), 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], tumor necrosis factor (TNF), prostaglandins (PGs), parathyroid hormone (PTH) and parathyroid hormone-related protein promoter (PTHrP), causing osteoblasts/stromal cells, osteocytes, and lymphocytes to generate macrophage colony-stimulating factor (MCSF) and receptor for activation of nuclear factor kappa B (NF-κB) (RANK) ligand (RANKL) that ultimately enable osteoclast precursors to differentiate into mature bone-resorbing osteoclasts (Hicks, 2006). Bone resorption by osteoclasts is consisted of two processes, osteoclast differentiation and osteoclast bone-resorbing. The differentiation of osteoclasts is the multi-step process principally regulated by MCSF, RANKL and osteoprotegerin (OPG) (Teitelbaum, 2000). The molecular mechanism underlying osteoclast differentiation has been well defined today. M-CSF contributes to survival and proliferation of osteoclast precursors from the hematopoietic lineage through interaction with its receptor c-fms (Udagawa et al., 1990), while RANKL contributes to the differentiation of these precursors into mature osteoclasts through interacting with its receptor RANK (Dougall et al., 1999; Li et al.,
Besides, another RANKL receptor, osteoprotegerin (OPG), negatively regulates osteoclastogenesis by competing with RANK for RANKL binding (Kong et al., 1999; Hofbauer et al., 2000).

Osteoclast bone-resorbing is the multistep process in which differentiated osteoclasts adhere to bone, secrete acid and proteases that degrade bone in an extracellular compartment and remove digested materials through transcytosis. The initiation of osteoclast bone-resorbing activity is the plasma membrane reorganization of osteoclasts. Four distinct membrane domains, the sealing zone (SZ), the ruffled border (RB), the basolateral domain (BD) and the functional secretory domain (FSD) are formed (Coxon and Taylor, 2008), which ultimately make osteoclasts ready to resorb bone. During bone resorption by osteoclasts, these four membrane domains carry out their own function that are essential to osteoclast bone-resorbing. The fate and the functions of these four membrane domains are quite well known today. For example, the SZ is responsible for the bone attachment (Väänänen and Horton, 1995). RB is the place where the secretion of digestive acid and proteases take place (Coxon and Taylor, 2008; Li et al., 1999; Lange et al., 2006; Zhao and Väänänen, 2006). The FSD is responsible for excretion of degradation products delivered through transcytosis (Mulari et al., 2003; Salo et al., 1996). Moreover, the fate and the function of these four distinct membrane domains are linked. All the findings above are likely to guide us to attach great importance to the endosomal trafficking in osteoclasts. Normal osteoclast function is largely dependent on the endosomal pathway in osteoclasts. Ruffled border formation, extracellular acidification, transport and secretion of ions and proteases all
result from a well-developed and tightly-regulated “endosomal trafficking system” in osteoclasts.

**Mice deficient in osteoclast-specific genes**

Osteopetrosis is a genetic bone disease characterized by osteoclast failure and conceptually provides a model for understanding the role of osteoclasts in health and disease. Mouse models with osteopetrotic mutations provided a wealth of information about genes with involvement of osteoclast function. There are several well-known examples of mice with inactivating mutations of osteoclast-specific genes which result in osteopetrotic phenotypes. Cathepsin K deficient mice exhibit osteoclast- rich osteopetrosis with the disrupted microstructure and altered biomechanical properties of the bone, underscoring the role of bone-degrading protease in osteoclast function and bone remodeling (Saftig et al., 1998; Saftig et al., 2000). Additionally, osteoclast-derived Cathepsin K deficiency resulted in increased osteoblast function and bone formation (Lotinun et al, 2013). Similarly, TRAP-deficient mice exhibited a mild osteopetrosis accompanied with the altered microstructure of growth plates, which also underscored the role of bone-degrading protease in osteoclast function and bone remodeling (Hayman et al., 1996). Finally, Atp6i deficiency results in severe osteopetrosis accompanied with wider growth plates and discontinued cortical outline in mice (Li et al., 1999). Extracellular acidification was impaired in osteoclasts from Atp6i-deficient mice (Li et al., 1999), underscoring the role of acid in osteoclast function and bone remodeling.
Potential role of Snx10 in osteoclastic bone resorption

Vesicular trafficking of endosomal pathway is required for the intracellular transport of the endocytosed cargo, for the creation and the maintenance of the ruffled border, for delivery and secretion of ions and proteases to digest bone, and the transcytosis of digested materials (Coxon and Taylor, 2008; Nesbitt and Horton, 1997; Stenbeck and Horton, 2004). Accordingly, any resulting failure(s) of the normal vesicular trafficking will affect osteoclast function (Coxon and Taylor, 2008). For example, the gene *pleckstrin homology domain–containing family M member 1 (PLEKHM1)* was identified as a osteoclast function gene and required for vesicular transport. Osteoclasts from human patients and from rats deficient in Plekhm1, exhibit resorption activity failure, developing osteopetrosis (Van et al., 2007). Normal osteoclast function is largely dependent on the endosomal pathway in osteoclasts, however the mechanisms underlying which still remains largely undefined. It was known that different Rab GTPases exercise a regulatory function through the recruitment of specific effector proteins to the membrane where they are localized. For instance, rab5 and its effector Early Endosome Antigen 1 (EEA1) are found on early endosomes, and play a key role in early endosomal trafficking of the endocytic cargo (Bucci et al., 1992). The sorting nexin (SNX) family are involved in various aspects of protein sorting and trafficking (Woby and Dixon, 2007). All the members of the SNX family are unified by a common phospholipids-binding motif, the PX domain, rendering them able to form protein-protein complexes and protein-lipid interactions (Worby and Dixon, 2002). One SNX family member, SNX10, was found to mediate vacuolization in mammalian cells (Qin et al., 2006). Moreover, SNX10 is involved in ciliogenesis where Snx10 interaction with V-
ATPase is required in zebrafish, (Chen et al., 2012). Finally, the recently discovered Phosphatidylinositol 3-phosphate (Pi3P) binding, the cooperation between Snx10 and Pi3P shed light on a novel and emerging theme in the role of Snx10 in aspect of early endosomal homeostasis since Pi3P is specifically localized in early endosome (Qin et al., 2006; Yao et al., 2009). Previous studies in my lab show that Snx10 is strongly up-regulated during RANKL-induced osteoclast differentiation \textit{in vitro} and \textit{in vivo}, and is required for osteoclastic bone resorption \textit{in vitro} (Zhu et al., 2012). All these suggest that Snx10 activity might be involved in the osteoclast function through regulation of membrane trafficking and endosome homeostasis.

**Osteopetrorickets in humans**

As the most severe form of osteopetrosis, autosomal recessive osteopetrosis (ARO) was thought to be infant-onset, and it could lead to death if left untreated (Tolar et al., 2004; Segovia-Silvestre et al., 2009; Del Fattore, 2008; de Vernejoul and Kornak, 2010; Cleiren et al., 2001; Frattini et al., 2003; Waguespack et al., 2003; Letizia et al., 2004; Balemans et al., 2005; Sobacchi et al., 2013). Therefore ARO is also known as malignant infantile osteopetrosis. When rachitic phenotype is superimposed upon ARO, osteopetrorickets is expected. Osteopetrorickets, an autosomal recessive condition where osteopetrosis and rickets occur simultaneously, is characterized by excessive bone mass and failure of skeletal mineralization (Demirel et al., 2010). X-ray test is the most frequent tool for diagnosis with an osteopetrorickets case. Bone bending and/or metaphyseal cupping/fraying in addition to “bone in bone” appearance will be a direct proof for diagnosis. The serum level of calcium and parathyroid hormone (PTH) also
need to be considered. For osteopetrorickets, it is a paradoxical association because it occurs in the context of a positive total body calcium balance. Traditionally, it was thought that rickets in osteopetrorickets cases was due to osteoclast failure, given that since more than 99% of the calcium resides in the skeleton, there is a net decrease in serum calcium and therefore the calcium deficiency leaves the newly formed osteoid unmineralized (Kaplan et al., 1993; Kirubakaran et al., 2004). A recent study in mice and humans challenges this notion and puts forward the following provocative hypothesis: osteoclast dysfunction alone cannot be responsible for the imbalance in calcium homeostasis that leads to mineralization defects in rickets (Schinke et al., 2009). The authors demonstrate that rickets is multifactorial due to both impaired gastric acidification and the inability of osteoclasts to resorb bone (Schinke et al., 2009).
References


CHAPTER 2

Requirement of Osteoclast-derived Snx10 for Osteoclast to Form Ruffled Borders and Resorb Bone
Abstract

To study the role of osteoclast-derived Snx10 in osteoclastic bone resorption in vivo, Snx10 was deleted specifically in the osteoclast lineage by crossing mice with floxed Snx10 alleles with Cathepsin K-Cre mice. These osteoclast-specific Snx10-deficient mice (OC Snx10-deficient) exhibited a dramatic increase in bone mineral density (BMD) and bone mineral content (BMC), suggestive of a typical osteopetrosis phenotype. Micro-CT analysis showed that long bones of OC Snx10-deficient mice have marrow cavities filled with excessive bone mass. OC Snx10-deficient had significantly higher BV/TV, higher trabecular number and significantly reduced trabecular spacing than controls. Trabecular thickness values, on the other hand, were not significantly different between OC Snx10-deficient and controls. Hematoxylin/Eosin and Von Kossa staining of undecalcified femur sections confirmed the presence of unresorbed bone mass within the bone marrow space, demonstrating osteopetrosis phenotype in OC Snx10-deficient mice. Van gieson staining and relative histomorphometric analysis showed that the skeletal mineralization were not significantly different between OC Snx10-deficient mice and controls, suggesting that osteoclast-derived Snx10 was not involved in skeletal mineralization. Osteoclast-derived Snx10 deficiency did not completely inhibit osteoclast formation, however, resulted in reduced resorption capacity, defective extracellular acidification, vesicular transport to the ruffled border and ruffled border formation of osteoclasts. Taken together, these data showed that osteoclast-derived Snx10 is required for osteoclastic bone resorption through vesicular trafficking of endosomal pathway in vivo.
Introduction

Normal osteoclast function is largely dependent on the endosomal pathway in osteoclasts. The mechanisms underlying osteoclast endosomal trafficking are not completely defined. Different Rab GTPases exercise a regulatory function through the recruitment of specific effector proteins to the membrane where they are localized. For instance, rab5 and its effector Early Endosome Antigen 1 (EEA1) are found on early endosomes, and play a key role in early endosomal trafficking of the endocytic cargo (Bucci et al., 1992). GTP-bound rab5 specifically interacts with numerous cytosolic effector proteins, including the phosphatidylinositol 3-kinase (Pi3K) (Christoforidis et al., 1999), securing the local generation of Phosphatidylinositol 3-phosphate (Pi3P), which in turn will direct recruitment of effector proteins with affinity for Pi3P. Sorting nexins all contain a common phospholipid-binding motif (the PX domain), which mediate protein-PiP interactions. PX domains of Snx proteins determine phospholipid binding specificity (Yao et al., 2009). The recently discovered cooperation between Rabs and SNX proteins represents a novel and emerging theme in vesicular transport, a process that is common to many different cell systems (Steinberg et al., 2013; Pfeffer, 2013). One of the prerequisites for osteoclasts to resorb bone is the normal vesicular transport in osteoclasts (Coxon and Taylor, 2008). For example, osteoclasts from human patients and from rats deficient in Plekhm1, a protein with a critical function in vesicular transport in osteoclasts, exhibit inhibited bone resorption capacity (Van et al., 2007).

One SNX family member, SNX10, was found to mediate vacuolization in mammalian cells (Qin et al., 2006). Moreover, in zebrafish, SNX10 was found to be
involved in ciliogenesis where Snx10 interaction with V-ATPase is required (Chen et al., 2012). During investigations of genes differentially expressed during RANKL induced osteoclast differentiation at my lab, previous studies showed that Snx10 was very strongly upregulated both in vitro and in vivo (Zhu et al., 2012). Snx10 expression was found to be localized in osteoclasts (Zhu et al., 2012). Snx10 silencing inhibited formation of resorption pits on hydroxyapatite (Zhu et al., 2012). Taken together, these results indicate that Snx10 is required for osteoclastic bone resorption in vitro and might be involved in intracellular trafficking in the osteoclasts.

Autosomal recessive osteopetrosis is a heterogenous genetical disease characterized by osteoclast failure and early childhood mortality. Osteoclast failure can be due to impaired osteoclast differentiation as evidenced by reduced osteoclast number and/or defective osteoclast function as evidenced by normal or elevated number of osteoclasts failing to resorb bone. To date, mutations in several genes have been demonstrated to contribute to pathogenesis of human disease. Mutations in genes including RANK (Nakagawa et al., 1998; Hsu et al., 1998) and RANKL (Anderson et al., 1997; Tsurukai et al., 1998) affect osteoclast differentiation. Mutations in genes including ATP6i (Li et al., 1999), Clc-7 (Lange et al., 2006), Ae2 (Josephsen et al., 2009), Cathepsin K (Saftig et al., 1998), TRAP (Hollberg et al., 2002) and PLEKHM1 (Van et al., 2007) affect osteoclast function. However, those unidentified genes involved in human osteopetrosis are still the mystery today. Snx10, although was found to be expressed in osteoclasts and required for osteoclastic bone resorption in vitro (Zhu et al., 2012), a role for Snx10 in osteoclast function in vivo as well as the mechanism/s by which Snx10 regulates osteoclastic bone resorption remains largely uncharacterized.
The overall goal of this chapter is to characterize the role of Snx10 in bone osteoclastic bone resorption *in vivo.*
Materials and methods

Generation of mouse models

We obtained the Snx10 targeting vector, PG00216_Z_2_C06, from the European Conditional Mouse Mutagenesis Program (EUCOMM). This vector is a “knockout first” gene trap (see Figure 2.1 A) which inserts a flippase site-flanked Neo selection cassette with an IRES and LacZ reporter into intron 3 and inserts LoxP sites flanking exons 4 and 5. Exons 4 and 5 contain the functional domain of Snx10, the PX domain required for phospholipid interactions. This allele is designated Snx10\textsuperscript{tm1a(EUCOMM)Hmgu}. Hereafter in this report, we refer to the resulting targeted allele as Snx10\textsuperscript{Neo-f}.

The vector was electroporated into V6.5 ES cells. Neomycin resistant clones were picked, expanded and screened for correct insertion by Long Range Genomic PCR using the following vector-specific primers and gene-specific primers:

5' Integration
Gene Specific Forward (GF3). 5'-GCTTATGGTCGACTCATCGGAGAATC-3'
LacZ Reverse (LAR7), 5'-GGTGTGGGAAAGGGTTCGAAGTTCCTAT-3'
Amplicon size=5,170 bp

3' Integration
LacZ Forward (LAF), 5'-GAGATGGCGCAACGCAATTAATG-3'
Gene Specific Reverse (GR4), 5'-CACAGAAGTAATGTACGCTAATGGCAACG-3'
Amplicon size=5,680 bp

The resulting clones were injected into host blastocysts to generate mouse chimeras. Two male chimeras were bred with C57BL/6J females. Germ line
transmission was confirmed by PCR, using primers flanking the third loxP site (LoxP3 Forward: 5'-ATAACTAACCAGGCAAACA-3' and LoxP3 Reverse: 5'-TTGTCAAGTCCGTGTGCGTGCGTGCGTGCGT-3'). Snx10Neo-f/+ offspring were bred to homozygosity to generate animals for experiments at the expected Mendelian ratio of approximately 25%. PCR genotyping using the preceding primer pair produced bands of 213 bp for the WT (+/+), 273 bp for the Snx10Neo-f/Neo-f, and both bands for the heterozygotes Snx10Neo-f/+.

Osteoclast-specific Snx10-deficient mice were generated in two steps. First, the Neo cassette was removed by crossing Snx10Neo-f/+ males with females homozygous for Rosa26-driven FLP recombinase (strain B6.129S4-Gt(ROSA)26 Sortm1(FLP1)Dym/RainJ) (Farley et al., 2000), resulting in an allele with exons 4 and 5 flanked by loxP sites (Snx10fl/+). Snx10fl/fl animals were viable and fertile. Then, homozygous males were crossed with females heterozygous for Cre recombinase driven by the cathepsin K promoter (Nakamura et al., 2007) for breeding of Catk;Snx10fl/+ males which were backcrossed with Snx10fl/+ females. The resulting mice carry an osteoclast-specific Snx10 allele with exons 4 and 5 deleted, and therefore a null allele, which we designate Snx10OC-. Snx10OC-;OC- homozygous animals are hereafter referred to as “OC Snx10-deficient”, for osteoclast specific knockout. Animal procedures used in this study were approved by IACUC of The Forsyth Institute.
In vivo measurement of bone mineral content (BMC) and bone mineral density (BMD) of the whole skeleton

Mice were anesthetized with a combination of ketamine (80mg/kg) and xylazine (10mg/kg) intramuscularly for complete sedation. Dual Energy X-ray Absorptiometry (DEXA) analysis was performed using a Lunar PIXImus Scanner (Lunar PIXImus2, software version 1.4X). The values for bone mineral content (BMC) and bone mineral density (BMD) of whole skeleton were obtained.

Analysis of X-Ray and Micro CT

X-rays of anesthetized mice were performed using a cabinet X-Ray system (Model 43855A, HEWLETT hp PACKARD) with the high-speed holographic film (Kodak) at 40 kV. For micro CT, mice were sacrificed by CO₂ asphyxiation. Bone samples were then dissected and fixed in 4% paraformaldehyde (PFA) for 18 hours, followed by 70% ethanol, then stored at 4 degrees until scanned. Samples were scanned (μCT 40, Scanco Medical) for generation of 3D reconstruction of the sagittal plane and surface plane, and the parameters BV/TV (bone volume per tissue volume), Tb.N (trabecular number), Tb.Th. (trabecular thickness) and Tb.Sp (trabecular spacing) were analyzed and obtained.

Isolation of splenocytes and osteoclast differentiation

We performed osteoclast differentiation with splenocytes as source of osteoclast precursors in that long bones of OC Snx10-deficient mice have limited marrow cavity and could not flushed. Spleens were dissected, placed into a cell strainer (BD Falcon*),
Mesh Size: 40µm) and mashed through the mesh into a conical tube (50mL) using a syringe plunger. The cell strainer was then rinsed with alpha-MEM/10% FBS followed by splenocytes collection and counting. To induce osteoclast differentiation, splenocytes were plated onto a 24-well plate (1 x 10⁶ cells/well) in alpha-MEM /10% FBS. M-CSF (25 ng/ml) and RANKL (50 ng/ml) were added. Cells were cultured for totally 7 days with changing of medium and cytokines every other day.

**TRAP staining of cells**

After splenocytes were differentiated into osteoclasts using the method above, cultures were washed with PBS, fixed first in 4% PFA, then in ethanol/acetone, and air dried for 2 minutes. Cells were then incubated in TRAP staining solution (Fast Red Violet LB Salt/ Napthol AS-MX phosphate) at 37°C until the color developed (20 minutes to 1 hour). The wells were washed with PBS again, then air-dried and finally photographed under a light microscope (Leica, DM LS).

**Pit formation assay and scanning electron microscopy (SEM)**

Spleen was dissected and splenocyte were isolated and then plated on 24-well Osteo Assay Surface plates (Corning) (1 x 10⁶ cells/well) in alpha-MEM medium supplemented/10% FBS with M-CSF (25 ng/ml) and RANKL (50 ng/ml). The osteoclast differentiation with splenocytes as osteoclast precursors was performed as described above. After mature osteoclasts form, cultures were processed with 10% bleach solution to remove all cells, followed by washing in PBS for 1 min and fixing in solution (4% (w/v) PFA / 1% (v/v) glutaraldehyde diluted in 0.1 M phosphate buffer) for 5 min,
After rinsing and drying, the wells were then sputter-coated with gold (Denton Vacuum Desk V) and finally visualized and photographed with SEM (ZEISS LS10 EVO). The area of resorption pits was quantified using Image J (National Institutes of Health, Bethesda, MD).

Assessment of extracellular acidification

Spleens were dissected and splenocytes were cultured on dentin slices in alpha-MEM medium /10% FBS supplemented with M-CSF (25 ng/ml) and RANKL (50 ng/ml), to induce osteoclast differentiation. The osteoclast differentiation with splenocytes as osteoclast precursors was performed as described above. After differentiated osteoclasts form, aridine orange (5ug/ml) was added to the differentiated osteoclasts cultures followed by the incubation at 37°C for 15 min, and the resulting cultures were washed with PBS and then chased for 15 min (Wu et al., 2008; Li et al., 1999). The Laser Scanning Confocal Microscope (Leica, SP5X) was used to visualize the acidified compartment of osteoclast (490-nm excitation filter; 525-nm arrest filter). Images were obtained.

Histology

Bone samples from mice were dissected, then fixed in 4% PFA for 18-24 hours and finally stored in 50% alcohol at room temperature. For paraffin embedding, the bone samples were washed with 5, 10, and 15% glycerol in PBS, each for 15 min, followed by decalcification with 10% EDTA / 0.1M TRIS for 2 weeks. After decalcification, bone samples were embedded in the low melting paraffin. Resulting
embedded bone samples were sectioned (5-micron intervals). Decalcified paraffin sections were used for H&E, IF and TRAP staining. For plastic embedding, the bone samples were dehydrated in a graded series of ethanol and then infiltrated in the methyl and butyl methacrylate resin. The resulting bone samples were embedded in, again, the methyl and butyl methacrylate resin. Embedded samples were bisected with a precision saw. Obtained segments at the transverse planes and the longitudinal planes were sectioned with the microtome (Leica RM2165) and collected onto coated slides. The resulting slides were press-mounted and then dried in an oven at 50°C for hours. Slides were deplasticized with xylenes and rehydrated with ethanol of decreasing concentrations, followed by Von Kossa-Van Gieson’s staining. The resulting micro-ground sections were mounted onto plastic slides, and then polished using the EXAKT CS400 system. The final thickness should be no more than 80 microns. Finally the micro-ground slides were etched in a solution of 50% acetone and 50% dehydration alcohol, followed by rinse in deionized water and Von Kossa- Van Gieson’s staining.

**Morphometric analysis**

Five images (magnification: 40X) below the metaphyseal growth plate of the distal femur were obtained with Olympus MicroSuite Biological Suite™. Calibrated images were assessed for quantification of trabecular area (mm²), osteoid area (mm²) and ratio of osteoid area (mm²) to total trabecular area (mm²) via thresholding (HSI – hue, saturation and intensity).
TRAP staining of bone sections

After deparaffinization with xylene and rehydration with ethanol of decreasing concentrations, the bone sections were incubated in TRAP staining solution (Fast Red Violet LB Salt/ Naphthol AS-MX phosphate) at 37°C until the color developed (20 minutes to 40 minutes). The resulting sections were counter stained with Fast Green (Santa Cruz), air-dried, sealed with coverglass and photographed under a light microscope.

Immunofluorescence

After deparaffinization with xylene and rehydration with ethanol of decreasing concentrations, antigen retrieval was performed for 10 min in boiling 10mM Na Citrate (Ph=6) in microwave. After cooling down, sections were in water for 10 min and then in 2% Sodium Borohydride in PBS for 1.5 hours. Washing with PBS 5 times were followed by block in 2% bovine serum albumin (BSA) in PBS for 1 hour. Resulting sections were finally incubated with primary antibodies (Santa Cruz SC-104657, 1:200) overnight, washed in PBS 3 times and incubated with secondary antibodies (Alexa Flour 488, Molecular Probes by Life Technology, A-11055, 1:500) for 1 h. Images were obtained using the fluorescence microscopy (Zeiss).

Transmission electron microscopy

Mice were perfused with solution (4% (w/v) PFA / 1% (v/v) glutaraldehyde diluted in 0.1 M phosphate buffer). Bone samples were dissected and immediately fixed in the same solution as what perfusion used above at 4°C for 1 h. After fixation, bone samples were cut into thin slices. The resulting slices continued to be fixed for additional 24 h
and then rinsed in 0.1M sodium phosphate buffer. Bone slices were decalcified in 10% (w/v) EDTA in PBS for 3 days, post fixed in 1% (v/v) OsO4 and embedded in epon, followed by ultrathin sectioning. The resulting sections were stained with uranyl acetate and lead citrate, and then visualized using a Transmission Electron Microscopy (FEI Tecnai G2 Spirit).

REAL-TIME PCR

RNA was extracted from the femur, the brain, the stomach and differentiated osteoclasts with Trizol reagent (Invitrogen). Reverse transcription was performed using a Reverse Transcription Kit (Qiagen). PCR was performed according to standard protocols with the following primers:

SNX10 (Forward): 5’ GAACAATCGCCAGCATGTCGAC-3’
SNX10 (Reverse): 5’-ATGTCTCCTGGAGTTGTCAGATG-3’
SYBR®Green Master mix (Bio-Rad) was used.

Statistical analysis

Data of values were analyzed using Student’s t-test. Significant difference was considered if P<0.05.
Results

Consequences of Osteoclast-derived Snx10 deficiency in vivo: Osteopetrosis

OC Snx10-deficient mice showed a significant reduction in Snx10 expression at mRNA level in osteoclasts (0.010+/-.016 vs 1.176+/-.0121, respectively, n=3 per group, **p< 0.001, Figure 2.2 A) and femur bone(0.096+/-.021 vs 1.002+/-.098, respectively, n=3 per group, **p< 0.001, Figure 2.2 B); however Snx10 expression of other sites including brain (0.934+/-.0145 vs 0.987+/-.0117, respectively, n=3 per group, p= 0.641, Figure 2.2 C) and stomach (1.011+/-.029 vs 1.020+/-.0288, respectively, n=3 per group, p=0.316, Figure 2.2 D) was not affected, confirming osteoclast-specific deficiency of Snx10. OC Snx10-deficient mice were viable and survive until 5th month post-partum, however, exhibited mild growth retardation (Figure 2.3), accompanied with failure of tooth eruption by 3 weeks of age (Figure 2.4 A, B), compared with controls, suggestive of high bone mineral density in jaws of OC Snx10-deficient mice.
Figure 2.1 Generation of osteoclast-specific Snx10-deficient mice (OC Snx10-deficient)

Figure 2.2 Snx10 relative expression at mRNA level in different sites
(A) qPCR analysis of RNA from osteoclasts showed significant reduction in Snx10

(A) **Figure 2.2 Snx10 relative expression at mRNA level in different sites**

(A) qPCR analysis of RNA from osteoclasts showed significant reduction in Snx10
(Figure 2.2 continued) expression in osteoclasts of OC Snx10-deficient mice.
(B) qPCR analysis of RNA from femur bone showed significant reduction in Snx10 expression in femur bone of OC Snx10-deficient mice.
(C) qPCR analysis of RNA from brain showed Snx10 expression was not affected in brain of OC Snx10-deficient mice.
(D) qPCR analysis of RNA from brain showed Snx10 expression was not affected in stomach of OC Snx10-deficient mi
Figure 2.3 Appearance of OC Snx10-deficient mice and control

Figure 2.4 Teeth eruption of OC Snx10-deficient mice and control

(A) Oral exam shows OC Snx10-deficient mice had failure of teeth eruption at day 21.
(B) X-ray exam shows OC Snx10-deficient mice had failure of teeth eruption at day 21.
X-ray analysis showed higher radio-density in OC Snx10-deficient mice (Figure 2.5). Analysis of BMD by DXA demonstrated that the whole body BMD was significantly higher in 6-week-old OC Snx10-deficient mice compared to controls (0.0667 +/- 0.004 g/cm² vs. 0.0516 +/- 0.003 g/cm², respectively, n=3 per group, **p<0.01) (Figure 2.6 A). DXA analysis of whole body BMD of 9 week-old mice also revealed a significant increase in OC Snx10-deficient mice compared to controls (0.074 +/- 0.0022 g/cm² vs. 0.057 +/- 0.0025 g/cm², respectively, n=8 per group, **p <0.01) (Figure 2.6 B). Micro-CT analysis exhibited the long bones of OC Snx10-deficient mice had marrow cavities filled with excessive bone mass (Figure 2.7 A). OC Snx10-deficient mice had significantly higher bone volume per tissue volume (0.461 +/- 0.046 vs 0.067 +/- 0.006, **p< 0.001), higher trabecular number (18.131 +/- 1.665 1/mm vs 2.384 +/- 0.514 1/mm, **p< 0.001) and significantly reduced trabecular spacing (0.045 +/- 0.004 mm vs 0.3223 +/- 0.044mm, **p< 0.001) than controls (Figure 2.7 B, C, D). Trabecular thickness values, on the other hand, were not different (0.039 +/- 0.008 mm vs 0.0317 +/- 0.004 mm, p= 0.235, Figure E). Hematoxylin/Eosin (Figure 2.8 A) and Von Kossa staining (Figure 2.8 B) of femur sections from OC Snx10-deficient mice confirmed the presence of excessive bone mass within the bone marrow space. Immunofluorescence showed that Snx10 expression at protein level existed in femur of controls, while Snx10 expression at protein level down-regulated in femur of OC Snx10-deficient mice (Figure 2.9 A, B). Taken together, these data showed that osteoclast-specific Snx10 deficiency resulted in osteopetrosis in vivo.
Figure 2.5 X-ray exam of the whole skeleton

Figure 2.6 Assessment of the whole body BMD using Dual Energy X-ray Absorptiometry

(A) 6-week-old OC Snx10-deficient mice had a significantly increased whole body BMD, compared with controls.

(B) 9-week-old OC Snx10-deficient mice had a significantly increased whole body BMD, compared with controls.
Figure 2.7 Micro-CT analysis of bone samples
(A) 3D reconstruction of sagittal plane of femur from OC Snx10-deficient mice showed excessive bone mass filling bone marrow cavity, compare to the controls.
Figure 2.7 (continued)

(B) OC Snx10-deficient mice had significantly higher trabecular number than controls (18.131 +/- 1.665 1/mm vs 2.384 +/- 0.514 1/mm, respectively, n=3 per group, **p< 0.001).

(C) OC Snx10-deficient mice had significantly higher BV/TV than controls (0.461 +/- 0.046 vs 0.067 +/- 0.006, respectively, n=3 per group, **p< 0.001).

(D) OC Snx10-deficient mice had significantly reduced trabecular spacing than controls (0.045 +/- 0.004 mm vs 0.3223 +/- 0.044mm, respectively, n=3 per group, **p< 0.001).

(E) Trabecular thickness were not different (0.039 +/- 0.008 mm vs 0.0317 +/- 0.004 mm, respectively, n=3, p= 0.235) between OC Snx10-deficient mice and controls.
Figure 2.8 Histology of bone

(A) Hematoxylin/Eosin staining of femur sections showed OC Snx10-deficient mice had excessive bone mass filling with marrow space.
(B) Von Kossa staining of undecalcified femur sections showed OC Snx10-deficient mice had excessive bone mass compared with controls.
Figure 2.9 Immunofluorescence microscopy of Snx10 expression at protein level in bone

(A) Immunofluorescence microscopy of Snx10 expression at protein level in bone (mag: 10X10) showed Snx10 expression was down-regulated in bone of OC Snx10-deficient mice, compared to controls.

(B) Immunofluorescence microscopy of Snx10 expression at protein level in bone (mag: 40X10) showed Snx10 expression was down-regulated in osteoclasts (blue arrows) in bone of OC Snx10-deficient mice, compared to controls.
Osteoclast-derived Snx10 deficiency doesn’t affect normal mineralization in vivo

Von Kossa/van Gieson staining of undecalcified femur sections showed that excessive mineralized bone mass filling marrow space in OC Snx10-deficient mice (Figure 2.10 A, B). Trabecular area in OC Snx10-deficient mice was significantly increased compared to controls (0.08617+/−0.0071 mm² vs 0.04138+/−0.00978 mm², respectively, n=3 per group, **p<0.001), further confirming osteopetrosis in OC Snx10-deficient mice (Figure 2.10 D). The osteoid area (0.00207+/−0.00089 mm² for OC Snx10-deficient vs 0.00185+/−0.00022 mm² for controls, n=3 per group, p=0.7066) and osteoid/trabecular area ratio (2.47477+/−1.28984% for OC Snx10-deficient vs 4.63433+/−1.12541% for Controls, n=3 per group, p=0.0942) did not vary significantly between OC Snx10-deficient mice and controls, confirming that OC Snx10-deficient mice are osteopetrotic with normal mineralization (Figure 2.10 C, E).
Figure 2.10 Mineralization assay of bone

(A) Von Kossa/ Van Gieson staining of undecalcified femur sections (metaphyseal) showed OC Snx10-deficient mice have excessive mineralized bone mass compared to controls.

(B) Von Kossa/ Van Gieson staining of undecalcified femur sections (diaphyseal) showed OC Snx10-deficient mice have excessive mineralized bone mass compared to controls.

(C) Morphometric analysis showed that the osteoid area (n=3, p=0.23) did not differ significantly between controls and OC Snx10-deficient mice confirming that OC Snx10-deficient mice were with normal mineralization.

(D) Morphometric analysis showed that OC Snx10-deficient mice had significantly increased trabecular area (n=3, *p<0.05) compared with controls confirming that OC Snx10-deficient mice were osteopetrotic.

(E) Morphometric analysis shows that Osteoid/trabecular area ratio (n=3, p=0.275) did not differ significantly between control and OC Snx10-deficient mice, confirming that Snx10 OC KO mice were osteopetrotic with normal mineralization.
Figure 2.10 (continued) Mineralization assay of bone
Snx10 deficiency doesn’t completely inhibit osteoclast formation, but inhibits osteoclastic bone resorption

TRAP staining of femur sections showed that bones of both OC Snx10–deficient mice and controls had TRAP-positive osteoclasts, indicating that Snx10 deficiency doesn’t completely inhibit osteoclast formation in vivo, however, osteoclasts of OC Snx10-deficient mice failed to form resorption lacunae (Figure 2.11 A), suggesting that bone resorption capacity was affected in OC Snx10-deficient mice. Due to limited bone marrow in osteopetrotic OC Snx10-deficient mice, we used splenocytes as a source of osteoclast precursors from OC Snx10-deficient mice and controls for ex vivo differentiation experiments. Splenocytes from OC Snx10-deficient mice also gave rise to TRAP-positive multinucleated osteoclasts ex vivo(Figure 2.11 B), further confirming that osteoclast-derived Snx10 deficiency did not completely inhibit osteoclast formation.
Figure 2.11 Osteoclastogenesis and resorption assay
(A) TRAP staining of femur sections showed that both OC Snx10-deficient mice and controls have TRAP-positive osteoclasts *in vivo*, however, osteoclasts of OC Snx10-deficient mice failed to form resorption lacunae (black arrows).
(B) TRAP staining of differentiated osteoclasts showed that splenocytes of both OC Snx10-deficient mice and controls could differentiate into TRAP-positive multinucleated osteoclasts.
(C) Pit formation assay showed that the osteo-surface was not resorbed completely by osteoclasts differentiated from splenocytes of OC Snx10-deficient mice.
(D) Pit formation assay showed that the total area of the osteo-surface resorbed by osteoclasts differentiated from splenocytes of OC Snx10-deficient mice was reduced significantly (**p<0.001**).
Figure 2.11 (continued) Osteoclastogenesis and resorption assay
Figure 2.11 (continued) Osteoclastogenesis and resorption assay
We performed pit formation assay and visualize the resulting pits with SEM, showing that the osteo-surface was not resorbed completely by osteoclasts differentiated from splenocytes of OC Snx10-deficient mice compared to the control (Figure 2.11C), although the osteoclasts differentiated from splenocytes of OC Snx10-deficient mice also gave rise to resorption pit (Figure 2.11C), confirming that osteoclast-derived Snx10 deficiency resulted in the reduced bone resorption capacity. Finally, the total area the osteo-surface resorbed by osteoclasts differentiated from splenocytes of OC Snx10-deficient mice was reduced significantly (Figure 2.11D) (0.35 +/- 0.09 mm$^2$ vs 2.44 +/- 0.43 mm$^2$, respectively, n=3 per group, **p<0.01). Taken together, these data demonstrated that osteoclast-derived Snx10 deficiency does not completely inhibit osteoclast formation, but results in reduced osteoclastic bone resorption capacity.

**Snx10 deficiency leads to impaired extracellular acidification of osteoclasts**

We cultured splenocytes from OC Snx10-deficient mice and controls on dentine slices for osteoclast differentiation. The resulting differentiated osteoclasts cultures were incubated with acridine orange for visualization of osteoclast acidification. Confocal microscopy revealed the presence of orange fluorescence indicating low pH in osteoclasts differentiated from splenocytes of controls while these disks were absent in osteoclasts differentiated from splenocytes of OC Snx10-deficient mice, indicating impaired extracellular acidification capacity of osteoclasts of OC Snx 10-deficient mice (Figure 2.12). As extracellular acidification of osteoclasts is responsible for degradation of the inorganic component of bone during osteoclastic bone resorption (Coxon and
Taylor, 2008), impaired extracellular acidification of osteoclasts from OC Snx10-deficient mice is in line with the observation that the capacity of osteoclasts from OC Snx10-deficient mice to resorb inorganic bone \textit{ex vivo} was decreased evidenced by resorption pit formation assay using Osteo Assay Surface plates.

Figure 2.12 Osteoclastic extracellular acidification assay
Snx10 deficiency results in impaired vesicular transport to the ruffled border in osteoclasts

Ultrastructural examination of bone sections by TEM revealed the intracellular vesicular transport to the ruffled border in osteoclasts of controls, while intracellular vesicular transport to the ruffled border was affected in osteoclasts of Snx10-deficient mice as evidenced by longer distance between vesicles and osteoclast ruffled border/bone border, less vesicles and enlarged vesicles (Figure 2.13 A, B).

Figure 2.13 Visualization of osteoclast vesicular transport (black arrows in Figure 2.12 A refer to osteoclast ruffled borders; black arrows in Figure 2.12 B refer to vesicles; scale bar: 2µm)
Snx10 deficiency results in impaired osteoclast ruffled border formation

Ultrastructural examination of bone sections by TEM demonstrated a well-developed ruffled border in osteoclasts of controls (Figure 2.13, black arrowheads) while a rudimentary ruffled border was observed in osteoclasts of OC Snx10-deficient mice (Figure 2.14, black arrowheads), suggesting osteoclast-derived Snx10 is involved in osteoclast ruffled border formation.

Figure 2.14 Visualization of osteoclast ruffled border formation (scale bar: 2um)
Discussion

Although it was known that osteoclast-derived Snx10 was involved in osteoclastic bone resorption \textit{in vitro}, the \textit{in vivo} role of osteoclast-derived Snx10 remains uncharacterized. In this study, Snx10 was deleted in the osteoclast lineage by crossing mice with the floxed Snx10 allele with CathepsinK-Cre mice, to generate osteoclast-specific Snx10-deficient mice (OC Snx10-deficient mice). OC Snx10-deficient mice exhibited extremely high bone mineral density and bone mineral content compared with controls, suggesting that osteoclast-specific Snx10 deficiency resulted in osteopetrosis \textit{in vivo}. OC Snx10-deficient mice showed excessive bone mass within the bone marrow space, further confirming osteoclast failure resulting from osteoclast-specific Snx10 deficiency \textit{in vivo}.

Either failure of osteoclast differentiation or the impaired osteoclast function contributes to osteopetrotic phenotype \textit{in vivo}. \textit{In vivo} histological analysis showed that long bones of OC Snx10-deficient mice also had TRAP-positive osteoclasts, combined with the \textit{in vitro} evidence that osteoclast precursors from OC Snx10-deficient mice could also differentiate into TRAP-positive multinucleated osteoclasts, suggested that osteoclast-derived Snx10 deficiency did not completely inhibit osteoclast formation. The total area of osteo-surface resorbed by osteoclasts differentiated from splenocytes of OC Snx10-deficient mice was reduced significantly compared with controls. All of these are in line with our \textit{in vivo} histological finds that osteoclasts of OC Snx10-deficient mice failed to form resorption lacunae. Therefore, we came to conclusion that osteoclast-derived Snx10 deficiency resulted in an impaired osteoclastic bone resorption capacity.
Osteoclasts are bone-resorbing cells. Bone resorption by osteoclasts largely depends on the formation of the extracellular acidified compartment of osteoclasts and acidified space between the ruffled borders and bone surface. Osteoclasts of osteoclast Snx10-deficient mice failed to form an acidic extracellular compartment evidenced by acridine orange staining, while which could be detected in osteoclasts of controls, demonstrating that osteoclast-derived Snx10 was involved in extracellular acidification of osteoclasts. The “acidification machinery” of osteoclast is consisted of vacuolar-type H+ -ATPase (V-ATPase), CLC7 chloride channel and anion exchanger 2 (AE2) and carbonic anhydrase II (CAII), all of which play an indispensable role in osteoclast extracellular acidification (Li et al., 1999; Wu et al., 2008; Josephsen et al., 2009; Lange PF et al., 2006; Van et al., 2007; Sly et al., 1983; Tolar et al., 2004). Disruption of any of “these parts” in “acidification machinery” abolished osteoclastic resorptive activity. For example, osteoclasts from mice deficient in Atp6i (also known as Tcirg1, which is an α3 subunit of V-ATPase) and from mice deficient in AE2, both of which are proteins with a critical function to form an acidic extracellular compartment, fail to resorb bone in vitro and in vivo (Li et al., 1999; Wu et al., 2008). ClC-7 deficient mice are osteopetrotic and osteoclastic extracellular acidification defect was detected in ClC-7 deficient mice (Kornak et al., 2001). Ostm1-deficiency also impaired osteoclast extracellular acidification due to failure of proper ClC-7 localization (Lange PF et al., 2006).

The formation of the ruffled border, an osteoclast organelle where the resorption takes place, is highly dependent on intracellular vesicular transport of osteoclasts (Coxon and Taylor, 2008). The fate and the function of intracellular vesicular transport, the ruffled border formation and the extracellular acidification are always linked.
Therefore, it is not surprising to speculate that the disruption of vesicular transport and/or the failure of the normal ruffled border formation might exist in osteoclasts that were found to suffer extracellular acidification defect. We found that both intracellular vesicular transport and ruffled border formation were impaired in osteoclasts of osteoclast Snx10-deficient mice, suggesting that osteoclast-derived Snx10 is involved both in the intracellular vesicular transport and the formation of both the ruffled border and the acidified compartment. Based on our preliminary data that Snx10-deficient osteoclasts have failure of early endosomal formation (not shown in this thesis), it is speculated that Snx10 mediates early endosomal formation and subsequent vesicular transport and ruffled border formation.

There are several well-known osteoclast-specific genes which are involved in osteoclast ruffled border formation. Disruption of ClC-7 (Kornak et al., 2001) or AE2 (Josephsen et al., 2009) in mice resulted in not only impairments of osteoclast extracellular acidification but also failure of normal osteoclasts ruffled border formation. Osteoclasts from human and rats deficient in Plekhm1 exhibited disrupted vesicular transport and ruffled border formation defect, since Plekhm1 was found to be indispensable for vesicular trafficking (Van et al., 2007). Osteoclasts from TRAP-deficient mice also exhibited failure of normal vesicular transport and ruffled border formation (Hollberg et al., 2002).

An osteopetrotic phenotype can be of either excessive mineralized bone or excessive unmineralized bone. The latter suggests impaired calcium homeostasis. For example, oc/oc mice with the disruption of Atp6i (also known as Tcirg 1) exhibit excessive unmineralized bone as evidenced by enrichment of osteoid and impaired
calcium homeostasis (Schinke et al., 2009). Radiological examination of the skeleton of oc/oc mice reveals high radio density in long bones as well as metaphyseal fraying and cupping (Schinke et al., 2009). In our study X-ray analysis of OC SNx10-deficient mice showed higher radio-density but no metaphyseal plate widening or metaphyseal fraying/cupping. Bone mineralization does not vary significantly between OC Snx10-deficient mice and controls, confirming that osteoclast-derived Snx10 is not involved in bone mineralization. Soon after we generated OC Snx10-deficient mice, mutations in SNX10 was identified in ARO patients (Aker et al., 2012; Megarbane et al., 2013; Pangrazio et al., 2013). The clinical manifestation and the lethality among these Snx10-dependent ARO patients varied, suggesting that the mutation(s) in Snx10 might be involved in other process(es) that are essential during development, in addition to osteoclast bone resorption.
References


CHAPTER 3
Requirement of Snx10-dependent Osteoclastic and Gastric Function for Bone and Calcium Homeostasis
Abstract

We found that Snx10, a molecule expressed in osteoclast, was also expressed in the stomach. Global Snx10-deficient mice had increased BV/TV, increased trabecular number, and reduced trabecular spacing, suggestive of an osteopetrotic phenotype. However, BMD and BMC were reduced in global Snx10-deficient mice. X-rays revealed metaphyseal fraying /cupping and rachitic widening of the growth plates, indicative of rickets superimposed on osteopetrosis, or “osteopetrorickets” in global Snx10-deficient mice. 3D reconstruction of surface plane of bones showed “moth-eaten” aspect, and histological analysis exhibited a discontinued cortical outline and wider growth plate in global Snx10-deficient mice. The van Gieson staining and relative histomorphometry revealed enrichment of non-mineralized osteoid on the surface of trabeculae in Global Snx10-deficient mice. Finally, Global Snx10-deficient mice were found to be hypocalcemic with a higher serum parathyroid hormone (PTH) level. Supplementation with calcium gluconate rescued the rachitic phenotype in global Snx10-deficient mice. These results demonstrated a combined phenotype in Global Snx10-deficient mice: osteopetrosis characterized by excessive bone mass and osteoclast failure, and rickets characterized by bone mineralization defect, rachitic widening of the growth plate and hypocalcemia.

Morphological examination of stomachs showed that global Snx10-deficient mice had abnormal stomachs, suggesting that Snx10 deficiency may result in a functionally impaired digestive system which might affect normal calcium absorption. Snx10 was also expressed specifically in gastric zymogenic cells. Immunofluorescence and H&E...
staining of stomach section showed that global Snx10-deficient mice exhibit much reduced zymogenic cell abundance. A significant increase in stomach pH of global Snx10-deficient mice was also detected. We concluded that gastric dysfunction with subsequent hypocalcemia leads to rickets in global Snx10-deficient mice.

Osteopetrorickets, the paradoxical association of insufficient mineralization in the context of a positive total body calcium balance, was thought to occur due to the failure of the osteoclasts to maintain normal calcium homeostasis. However, osteoclast-specific Snx10 deficiency had no effect on calcium balance, and therefore led to severe osteopetrosis without rickets. Our studies highlight the relationship between stomach and calcium bioavailability and its impact on bone health in the general population and provide novel insights into the mechanisms governing the regulation of bone accrual by the gastrointestinal tract.
Introduction

Rickets is the softening and bending of bones due to a failure in bone mineralization. It is accompanied with the low level of serum calcium and vitamin D deficiency. Rickets superimposed upon osteopetrosis (osteopetrorickets) is a condition the exact pathophysiology of which remains largely unknown. This is a paradoxical association because it occurs in the context of a positive total body calcium balance. However, because more than 99% of the calcium resides in the skeleton, there is a net decrease in serum calcium and therefore the calcium deficiency leaves the newly formed osteoid unmineralized (Kaplan et al., 1993). Therefore, traditionally, it was thought that rickets in osteopetrorickets cases was due to osteoclast failure (Kirubakaran et al., 2004; Kaplan et al., 1993).

As one of the largest tissues of the vertebrate body plan the skeleton does not function in isolation. The regulation of bone homeostasis by the gastrointestinal (GI) tract represents a remarkable example of a relationship between two systems that has not been fully appreciated until now. The recently discovered association of GI dysfunction with bone diseases defines a novel and emerging theme in bone biology, a process that is common to many different species. GI dysfunction serves as a negative effect on bone health as evidenced by the findings that a greater risk to develop osteopenia and osteoporosis was detected in Crohn’s disease (CD) patients (Pigot et al., 1992; Simbcrg et al., 1996; Jahnsen et al., 1997; Compston et al., 1987; Roux et al., 1995; Ghishan and Kiela, 2011). In IBD patients the high prevalence of low bone mass was found (Valentine and Sninsky, 1999; Roux et al., 1995; Andreassen at al., 1997).
Involvement of anorexia nervosa both in the retardation of skeletal development and the osteoporosis (Soyka et al., 1999; Howgate et al., 2013) further confirmed the importance of GI tract for normal bone homeostasis in another perspective. Finally, a high prevalence of osteopenia, the significantly low level of serum calcium and vitamin D deficiency in gastrectomized patients shed light on a previously unknown interrelationship of stomach, calcium homeostasis, and bone healthy (Zittel et al., 1997). Osteomalacia, the softening and bending of the bone due to the deficiency of calcium and vitamin D during adulthood, was found in gastrectomized patients (Clark et al., 1964; Eddy, 1971), which is a direct proof that there might be an association between stomach, calcium homeostasis and thus bone mineralization. Osteoporosis was found to be more frequent in old people with hypochlorhyria, which, again, attached great importance to the role of stomach in bone healthy, meanwhile further narrowed this down to a certain function of stomach, gastric acidification (Fourniera et al., 2009; Cole et al., 2008). The proton-pump inhibitor (PPI), a drug widely prescribed for acid-peptic disease, with blocking gastric acid production, have recently been found to be associated with hip fracture risk (Laine, 2009; Ngamruengphong et al., 2011). Therefore, it easily guides us to speculate that hypochlorhydria results in impaired calcium bioavailability thereby increasing fracture risk. All of these allow us to hypothesize new understanding of the interrelationship of any factor which might affect gastric acidification, calcium absorption and bone homeostasis.

In stomach pepsin and acid are responsible for dissolution of calcium salts in the food, which makes the calcium available to intestine for absorption. Proper calcium sufficiency is indispensable for normal bone mineralization. Disrupted calcium
homeostasis will cause skeletal mineralization defect. When this happens to osteopetrosis cases, osteopetrorickets will be expected. Therefore, defective mineralization superimposed upon osteopetrosis is the main histological evidence for the diagnosis of osteopetrorickets. Hypocalcemia itself is also one of the important clinical manifestations for diagnosis of osteopetrorickets. The value of alkaline phosphatase (ALP) and parathyroid hormone (PTH) in the serum are needed to be considered to provide an accurate picture of calcium homeostasis. Recognition and efficient correction of rickets and hypocalcemia are imperative for osteopetrorickets treatment including hematopoietic stem cell transplantation (HSCT) (Gonen et al., 2013; Kirubakaran et al., 2004; Kaplan et al., 1993).

Our lab previous reported that Snx10 was expressed in osteoclast, and required for osteoclastic resorption in vitro (Zhu et al., 2010). My current study with knockout mice model also confirmed that osteoclast-derived Snx10 deficiency leads to osteopetrosis in vivo and osteoclast-derived Snx10 is required for vesicular transport, ruffled border formation and extracellular acidification in osteoclasts. Soon after that, SNX10 mutations were discovered in ARO patients. One was a homozygous missense mutation causing an amino acid replacement in a highly conserved residue, R51Q (Aker et al., 2012) and one introduced an early stop codon (Megarbane et al., 2013). Besides, nine novel mutations (three nonsense mutations, three missense substitutions and three mutations affecting exon splicing) in SNX10 were then found in 14 ARO patients, and together, SNX10 mutations are now known to be accounting for about 4% of known ARO cases (Pangrazio et al., 2013). Some SNX10-dependent patients benefited from HSCT while some patients did not. For example, in one study (Pangrazio et al., 2013)
subject 5B diagnosed with ARO was also found to be hypocalcaemic. The subject received HSCT and experienced bone improvement, however died at the age of 18 months and multi-system organ failure was detected. This suggests that the transplant may have cured the osteoclast defect, but, osteoclast may not be the only site affected. Moreover, in one study (Megarbane et al., 2013) X-ray image of the osteopetrotic boy with identified stop mutation in SNX10 exhibited not only high radio density suggesting osteopetrosis but also bowed femur that is indicative of rickets, however, which was not investigated and fully explained. Therefore, ARO caused by Snx10 mutations is a genetically and phenotypically heterogeneous disease. The heterogeneous mutation(s) of SNX10 might affect other physiological processes including calcium homeostasis, finally leading to variable severity of clinical manifestations. The goal of this chapter is to characterize the role of Snx10 in bone homeostasis, and in the cross-regulation that might exist between bone and other organ/s.
Materials and methods

Targeting vector and mouse models

We obtained the Snx10 targeting vector, PG00216_Z_2_C06, from the European Conditional Mouse Mutagenesis Program (EUCOMM). This vector is a “knockout first” gene trap (Figure 3.1) which inserts into Intron 3 a flippase site-flanked Neo selection cassette with an IRES and LacZ reporter, as well as a spliced acceptor (SA) and poly A tail both of which lead to a premature termination of downstream transcription. Similar gene trap vectors were used (Doony et al., 2012; Ghazi-Noori et al., 2012; Li et al., 2011). Exons 4 and 5 contain the PX domain required for phospholipid interactions. This allele is designated Snx10\(^{tm1a(EUCOMM)Hmgu}\). Hereafter in this report, we refer to the resulting targeted allele as Snx10\(^{Neo-f}\). The gene-trap vector was electroporated into V6.5 ES cells. Neomycin resistant clones were picked, expanded and screened for correct insertion by Long Range Genomic PCR using the following vector-specific primers and gene-specific primers:

5' Integration
Gene Specific Forward (GF3). 5'-GCTTATGGTGCAGACTCATCGGAGAATC-3'
LacZ Reverse (LAR7), 5'-GGTGTGGGAAAGGGTTCGAAGTTCCTAT-3'
Amplicon size=5,170 bp

3' Integration
LacZ Forward (LAF), 5'-GAGATGGCGCAACGCAATTAATG-3'
Gene Specific Reverse (GR4), 5'-CACAGAAGTAATGTACGCTAATGGCAACG-3'
Amplicon size=5,680 bp
The resulting clones were injected into host blastocysts to generate mouse chimeras. Two male chimeras were bred with C57BL/6J females. Germ line transmission was confirmed by PCR, using primers flanking the third loxP site (LoxP3 Forward: 5’-ATAACTAACCAGGCAAACA-3’ and LoxP3 Reverse: 5’-TTGTCAAGTGCATGTGTGCGT-3’). Snx10\textsuperscript{Neo-f/+} offspring were bred to homozygosity to generate animals for experiments at the expected Mendelian ratio of approximately 25%.

PCR genotyping using the preceding primer pair produced bands of 213 bp for the WT (+/+), 273 bp for the Snx10\textsuperscript{Neo-f/Neo-f}, and both bands for the heterozygotes Snx10\textsuperscript{Neo-f/+}. Mice homozygous for this allele are severely deficient in Snx10 globally, so we hereafter refer to Snx10\textsuperscript{Neo-f/Neo-f} mice as “global Snx10-deficient,” for global Snx10 deficiency. This gene trap allele allowed us to generate a global SNX10-deficient mouse. Animal procedures used in this study were approved by IACUC of The Forsyth Institute.

**Osteoclast differentiation and lentivirus infection**

Isolation of splenocytes and osteoclast differentiation were performed as described in Chapter 2. For lentivirus infection, splenocytes (1 x 10^6 cells/well) were cultured overnight in alpha-DMEM/10% FBS supplemented with M-CSF (50ng/ml) in the regular 24-well plates and 24-well Osteo Assay Surface plates (Corning). The next day, the medium was replaced with lentiviral particle-containing medium in the presence of polybrene (6ug/ml) and cultured overnight. The following day, the culture medium was replaced with alpha-DMEM/10% FBS supplemented with M-CSF (25 ng/ml) and RANKL (50 ng/ml). Cells were cultured for additional 7 days with changing of medium and cytokines every other day.
REAL-TIME PCR and Western Blot

REAL-TIME PCR was performed as described in Chapter 2 of this thesis. Western blot analysis of protein extracted from differentiated osteoclasts was performed according to the standard protocols with an Anti-SNX10 antibody (Sigma, HPA015605). Beta-actin was used as a control.

Pit formation assay

Spleen was dissected and splenocyte were isolated and then plated on 24-well Osteo Assay Surface plates (Corning) (1 x 10^6 cells/well) in alpha-MEM medium /10% FBS supplemented with M-CSF (25 ng/ml) and RANKL (50 ng/ml). The osteoclast differentiation with splenocytes as osteoclast precursors was performed as described above. After mature osteoclasts form, cultures were processed with 10% bleach solution to remove all cells, followed by washing in PBS for 1 min and fixing in solution (4% (w/v) PFA / 1% (v/v) glutaraldehyde diluted in 0.1 M phosphate buffer) for 5 min. After rinsing and drying, the wells were then sputter-coated with gold (Denton Vacuum Desk V) and finally visualized and photographed with SEM (ZEISS LS10 EVO). The area of resorption pits was quantified using Image J (National Institutes of Health, Bethesda, MD).
**In vivo measurement of bone mineral content (BMC) and bone mineral density (BMD) of the whole skeleton**

Mice were anesthetized with a combination of ketamine (80mg/kg) and xylazine (10mg/kg) intramuscularly for complete sedation. Dual Energy X-ray Absorptiometry (DEXA) analysis was performed using a Lunar PIXImus Scanner (Lunar PIXImus2, software version 1.4X). The values for bone mineral content (BMC) and bone mineral density (BMD) of whole skeleton were obtained.

**Analysis of X-Ray and Micro CT**

X-rays of anesthetized mice were performed using a cabinet X-Ray system (Model 43855A, HEWLETT hp PACKARD) with a high-speed holographic film (Kodak) at 40 kV. For micro CT, mice were sacrificed by CO₂ asphyxiation. Bone samples were then dissected and fixed in 4% paraformaldehyde (PFA) for 18 hours, followed by 70% ethanol, then stored at 4 degrees until scanned. Samples were scanned (μCT 40, Scanco Medical) for generation of 3D reconstruction of the sagittal plane and surface plane, and the parameters BV/TV (bone volume per tissue volume), Tb.N (trabecular number), Tb.Th. (trabecular thickness) and Tb.Sp (trabecular spacing) were obtained and analyzed.

**Histology and immunofluorescence**

Bone samples from mice were dissected, then fixed in 4% PFA for 18-24 hours and finally stored in 50% alcohol at room temperature. For paraffin embedding, the
bone samples were washed with 5, 10, and 15% glycerol in PBS, each for 15 min, followed by decalcification with 10% EDTA / 0.1M TRIS for 2 weeks. After decalcification, bone samples were embedded in the low melting paraffin. Resulting embedded bone samples were sectioned (5-micron intervals). Decalcified paraffin sections were used for H&E, IF and TRAP staining. For plastic embedding, the bone samples were dehydrated in a graded series of ethanol and then infiltrated in the methyl and butyl methacrylate resin. The resulting bone samples were embedded in, again, the methyl and butyl methacrylate resin. Embedded samples were bisected with a precision saw. Obtained segments at the transverse planes and the longitudinal planes were sectioned with the microtome (Leica RM2165) and collected onto coated slides. The resulting slides were press-mounted and then dried in an oven at 50°C for hours. Slides were deplasticized with xylenes and rehydrated with ethanol of decreasing concentrations, followed by Von Kossa-Van Gieson’s staining. The resulting micro-ground sections were mounted onto plastic slides, and then polished using the EXAKT CS400 system. The final thickness should be no more than 80 microns. Finally the micro-ground slides were etched in a solution of 50% acetone and 50% dehydration alcohol, followed by rinse in deionized water and Von Kossa-Van Gieson’s staining.

Five images (magnification: 40X) below the metaphyseal growth plate of the distal femur were obtained with Olympus MicroSuite Biological Suite™. Calibrated images were assessed for quantification of trabecular area (mm²), osteoid area (mm²) and ratio of osteoid area (mm²) to total trabecular area (mm²) via thresholding (HSI – hue, saturation and intensity).
Stomach samples were dissected and immediately fixed in 4% PFA at 4°C overnight, followed by rinsing in 70% ethanol. The resulting bone samples were arranged in the agar (2%) in a tissue cassette, and then embedded with paraffin. Sections (5 μm) were cut, followed by deparaffinization and rehydration. Antigen retrieval was performed in boiling Trilogy solution (Cell Marque, Rocklin, CA). Blocking was performed in 1% BSA, 0.3% Triton X-100 in PBS. Resulting sections were incubated with primary antibodies overnight. Primary antibodies used were Snx10 (Santa Cruz SC-104657, 1:200), VEGF-B (Santa Cruz SC 1876, 1:250), and GIF (Washington University School of Medicine, 1:2000). GSII lectin and Hoechst 33358 labeling for secondary antibodies were used as described (Ramsey et al., 2007).

**Mechanical testing**

Tibias were dissected and then immersed in acrylic resin until solidification. The resulting samples were performed with mechanical testing (three-point-bending) to acquire the following parameters: maximal load, stiffness, energy to failure, and maximal displacement, which together provide a whole picture of the biomechanical plan of the bone.

**Measurement of gastric pH**

Measurement of gastric pH was performed as described (Langhans et al., 1997). Mice were fasted for 2 hours prior to the measurement. Under anesthesia as described in Chapter 2, the stomach was exposed after abdominal midline incision, followed by ligation of the pylorus and the esophagus. Saline solution was then injected into the
stomach. The fluid in the stomach was collected by a 1.5 mL Eppendorf Tube after an incision was performed on the stomach. The pH of the collected fluid was measured using a Star A321 pH Meter (Thermo Scientific Orion) equipped with a PerpHecT ROSS pH electrode with micro tip (Thermo Scientific Orion).

Determination of serum calcium and serum parathyroid hormone (PTH)

For analysis of calcium homeostasis, the level of serum calcium and serum PTH were measured. Blood was acquired by cardiac puncture and then collected in heparinized Eppendorf tubes. Serum was isolated by centrifugation at 3000 g for 10-15 min and stored at -80°C until use. The level of serum calcium was measured using QuantiChrom Calcium Assay Kit (DICA-500) (BioAssay Systems). The level of serum PTH was measured using Mouse PTH 1-84 ELISA Kit (Immutopics Inc).

Statistical analysis

Data of values were analyzed using Student’s t-test. Significant difference was considered if P<0.05.
Results

Global Snx10 deficiency results in osteopetrosis with low bone mineral density in vivo

We generated global Snx10-deficient mice with the gene trap vector (PG00216_Z_2_C06, Figure 3.1) from the European Conditional Mouse Mutagenesis Program (EUCOMM). This gene trap vector inserts into Intron 3 a flippase site-flanked Neo selection cassette with an IRES and LacZ reporter, as well as a spliced acceptor (SA) and a poly A tail both of which lead to a premature termination of downstream transcription. Similar gene trap vectors were used (Doony et al., 2012; Ghazi-Noori et al., 2012; Li et al., 2011). Exons 4 and 5 contain the PX domain required for phospholipid interactions. qPCR analysis revealed a significant decrease of Snx10 at mRNA levels in differentiated osteoclasts from the resulting global Snx10-deficient mice compared to the controls from the same litter (0.204 +/- 0.035 vs. 1.009 +/- 0.079, respectively, n= 3 per group, **p<0.001, Figure 3.2 A). Snx10 deficiency at the protein level was confirmed by Western blot (Figure 3.2 B). The total area resorbed by osteoclasts from global Snx10-deficient mice was significantly reduced (Figure 3.2 C) (0.26 +/- 0.15 mm² vs 3.99 +/- 1.41 mm², respectively, n=6, *p<0.05). Infection with SNX10 lentiviral particles reintroduced Snx10 expression at the protein level (Figure 3.2 C), and also rescued the pit formation defect (2.25 +/- 0.62 mm², n=6, p=0.008), further confirming that Snx10 deficiency resulted in the resorption defect (Figure 3.2 C).
Figure 3.1 Generation of global Snx10-deficient mice (G Snx10-deficient)
Figure 3.2 Confirmation of Snx10-deficiency in osteoclast

(A) qPCR analysis of RNA from osteoclasts showed a significant reduction in Snx10 expression at mRNA level in osteoclasts of global Snx10-deficient mice.

(B) Western blot analysis of protein from osteoclasts showed reduction in Snx10 expression at protein level in osteoclasts of global Snx10-deficient mice. Infection with a lentivirus expressing Snx10 reintroduced Snx10 expression at the protein level.

(C) The total area resorbed by osteoclasts from the resulting homozygous mice was significantly reduced. Infection with a lentivirus expressing Snx10 rescued the pit formation defect.
Global Snx10-deficient mice died between 3 and 4 weeks post-partum. By 14 days of age Global Snx10-deficient mice exhibited severe growth retardation (Figure 3.3), with failed tooth eruption (Figure 3.4 A, B) compared to controls. X-ray showed not only high radio density suggestive of osteopetrosis (Figure 3.5 A) but also metaphyseal cupping/fraying and rachitic widening of the growth plate (Figure 3.5 B) in the 3-week-old global Snx10-deficient mice, suggestive of rickets superimposed on osteopetrosis, compared with the controls. Further skeletal examination of 3 week-old mice by micro-CT (Figure 3.7 A) confirmed that the femur of Global Snx10-deficient mice were filled with excessive bone mass even in marrow cavities, consistent with a severe osteopetrosis phenotype. Global Snx10-deficient mice had significantly higher BV/TV (0.31 +/- 0.053 vs 0.071 +/- 0.005, respectively, n=3 per group, **p< 0.001), higher trabecular number (15.179 +/- 2.922 1/mm vs 2.299 +/- 0.004 1/mm, respectively, n=3 per group, **p< 0.001) and significantly reduced trabecular spacing (0.061 +/- 0.009 mm vs 0.392 +/- 0.042mm, respectively, n=3 per group, **p< 0.001) than controls. Trabecular thickness values, on the other hand, were not different (0.033 +/- 0.003 mm vs 0.032 +/- 0.004 mm, respectively, n=3 per group, p= 0.794). These values suggested that the observed phenotype was mainly the result of an osteoclast defect. But, analysis of the whole body BMD by DXA revealed that BMD was reduced ~ 14% in global Snx10-deficient mice compared to controls (0.0337 +/- 0.002 g/cm² vs. 0.0393 +/- 0.003 g/cm², respectively, n=6 per group, *p<0.05). Moreover, BMC was reduced ~ 43% in global Snx10-deficient mice compared to controls (0.114 +/- 0.043 g vs. 0.199 +/- 0.034 g, respectively, n=6, **p< 0.01).
Figure 3.3 Appearance of global Snx10-deficient mice and control at day 14

Figure 3.4 Teeth eruption of global Snx10-deficient mice and control at day 14
(A) Oral exam showed global Snx10-deficient mice had failure of teeth eruption at day 14, suggestive of high bone mineral density in jaws.
(B) X-ray exam showed global Snx10-deficient mice had failure of teeth eruption at day 14, suggestive of high bone mineral density in jaws.
Figure 3.5 X-ray exam of the whole skeleton
(A) X-ray showed higher radio density suggestive of osteopetrosis in the 3-week-old global Snx10-deficient mice compared with controls.
(B) X-ray shows not only high radio density but also metaphyseal cupping/fraying and rachitic widening of growth plate in the 3-week-old global Snx10-deficient mice, suggestive of rickets superimposed on osteopetrosis, compared with controls.
Figure 3.6 Assessment of bone mineral density (BMD) and bone mineral content (BMC) of the whole skeleton with Dual Energy X-ray Absorptiometry
(A) 3-week-old global Snx10-deficient mice exhibited decreased BMD of the whole skeleton, compared with controls.
(B) 3-week-old global Snx10-deficient mice exhibited significantly decreased BMC of the whole skeleton, compared with controls.
Figure 3.7 Micro CT analysis of bone

(A) Micro-CT 3D reconstruction of sagittal plane of femur from global Snx10-deficient mice showed excessive bone mass filling bone marrow cavity.

(B) Micro-CT 3D reconstruction of surface plane of femur from global Snx10-deficient mice showed “moth-eaten” aspect.
Global Snx10 deficiency in vivo results in rickets superimposed on osteopetrosis

Surface plane of 3D reconstruction by micro-CT showed that the femur surface of global Snx10-deficient mice exhibited “moth-eaten” appearance (Figure 3.7 B). Combined with the X-ray analysis which revealed metaphyseal fraying/cupping and rachitic widening of the growth plates in addition to high radio density in global Snx10-deficient mice (Figure 3.5 B), we speculated that rickets superimposed on osteopetrosis, or “osteopetrorickets” were in global Snx10-deficient mice. To confirm this, we compared global Snx10-deficient mice with global Atp6i (also known as Tcirg1)-deficient mice which was found to be osteopetrorickets with hypocalcaemia (Schinke et al., 2009; Li et al., 1999). Global Atp6i (also known as Tcirg1)-deficient mice were strikingly similar to global Snx10-deficient mice by X-ray (Supplemental Figure 3.1) and micro-CT (Supplemental Figure 3.2), with high radio density, metaphyseal cupping and fraying, rachitic widening of the growth plate and “moth-eaten” appearance, all characteristic of rickets.

These observations were further confirmed by histological analysis of the femur from 3-week-old global Snx10-deficient mice and their controls, which demonstrated: 1) a marrow space almost completely filled with excessive bone mass (Figure 3.8 A), 2) discontinuous cortical outline (black arrowheads) (Figure 3.8 A), in agreement with low BMD and BMC, as cortical bone contributes to most of weight of the skeleton, and 3) widening of the growth plate (Figure 3.8 B) in global Snx10-deficient mice. Moreover, von Kossa/van Gieson staining of undecalcified sections of femurs was performed and it revealed the presence of non-mineralized osteoid stained in pink in global Snx10-deficient mice (Figure 3.9 A). Relative histomorphometry confirmed a significant
increase in osteoid/trabecular ratio in global Snx10-deficient mice compared with controls (22.21+/−8.40% vs 2.72+/−1.24%, respectively, n=3 per group, *p<0.05, Figure 3.9 B), further confirming impaired mineralization in global Snx10-deficient mice. Taken together, these results demonstrated a combined phenotype in global Snx10-deficient mice: rickets characterized by bone mineralization defect and rachitic widening of growth plate, and osteopetrosis characterized by excessive bone mass.
Figure 3.8 Histology of bone

(A) Diaphyseal part of femur exhibited a marrow cavity almost completely filled with excessive bone mass and discontinuous cortical outline (black arrowheads) in global Snx10-deficient mice.

(B) Metaphyseal part of femur exhibited widening of the growth plate in global Snx10-deficient mice.
Figure 3.9 Mineralization assay of bone
(A) the Van Kossa/Van Gieson staining revealed the presence of non-mineralized osteoid on the surface of the trabeculae in global Snx10-deficient mice.
(B) Histomorphometry performed confirmed a significant increase in osteoid/trabecular ratio in global Snx10-deficient mice compared with controls.
Mechanical testing of tibial diaphyses (three-point-bending) was performed and the following parameters: maximal load, stiffness, energy to failure, and maximal displacement were acquired, providing the accurate information of the biomechanical properties of bone. Decreased maximal load ($p=0.003$), stiffness ($p=0.001$), energy to failure ($p=0.027$), while increased maximal displacement ($p=0.050$), were found in global Snx10-deficient mice compared to the contr (Table 3.1) demonstrating that the long bones of global Snx10-deficient mice are weaker, more softening and easier to deform in response to the mechanical stress. The results are consistent with the characteristics of rickets, softening and weakening of the bone, which makes bone easily bending. Taken together, Snx10 is required for normal biomechanical properties of bones.

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<th>p value</th>
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<td>Maximal displacement</td>
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<td>0.58 ± 0.1</td>
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Osteoclast-specific Snx10 deficiency in vivo results in osteopetrosis with no rickets

X-ray (Figure 3.10 A) and histological analysis (Figure 3.10 B) showed both OC Snx10-deficient mice and global Snx10-deficient mice had excessive bone mass compared with controls, but global Snx10-deficient mice had metaphyseal cupping/fraying and rachitic widening of growth plate while OC Snx10-deficient mice didn’t. Micro-CT 3D reconstruction of surface plane showed that “moth-eaten” aspect on bone of global Snx10-deficient mice is more severe compared with normal controls and OC Snx10-deficient mice (Supplemental Figure 3.3). The trabeculae were thoroughly mineralized and not covered by thick layers of unmineralized osteoid in both OC Snx10-deficient mice and normal controls, while unmineralized osteoid stained in pink were found in global Snx10-deficient mice (Figure 3.10 C). Assessment of serum calcium confirmed that global Snx10-deficient mice were hypocalcemic compared to normal controls, while serum calcium level did not differ between OC Snx10-deficient mice and normal controls (6.40 +/- 2.46 mg/dl for global Snx10-deficient vs 11.00 +/- 1.29 mg/dl for OC Snx10-deficient mice vs 11.89 +/- 1.33 mg/dl for controls, n=6 for global Snx10-deficient, n=6 for OC Snx10-deficient mice and n=12 for controls, ** p< 0.01, Figure 3.10 D). Taken together these data confirmed that Osteoclast-specific Snx10 deficiency only results in osteopetrosis with no rickets in vivo. Finally, we found severe pathological appearance of stomachs in global Snx10-deficient mice, while both OC Snx10-deficient mice and the controls have normal appearance of stomachs (Figure 3.10 E).
Figure 3.10 Osteoclast-specific Snx10 deficiency in vivo results in osteopetrosis with no rickets

(A) X-ray showed OC Snx10-deficient mice had higher radio-density of skeleton than control, but no metaphyseal cupping/fraying and rachitic widening of growth plate compared to global Snx10-deficient mice.

(B) Histological analysis showed global Snx10-deficient mice not only had excessive bone mass filling marrow space, but also exhibited discontinued cortical outline and growth plate widening, while OC Snx10-deficient mice exhibits excessive bone mass filling marrow space with a continued cortical outline and normal width of growth plate.

(C) The trabeculae were thoroughly mineralized and not covered by thick layers of unmineralized osteoid in both OC Snx10-deficient mice and control, while unmineralized osteoid stained in pink are found in global Snx10-deficient mice.

(D) Assessment of serum calcium confirmed that global Snx10-deficient mice were severely hypocalcemic compared to control, while serum calcium did not differ between OC Snx10-deficient mice and control.

(E) Severe pathological appearance of stomach in global Snx10-deficient mice were found.
Figure 3.10 (continued) Osteoclast-specific Snx10 deficiency in vivo results in osteopetrosis with no rickets
Figure 3.10 (continued) Osteoclast-specific Snx10 deficiency in vivo results in osteopetrosis with no rickets
Gastric dysfunction with subsequent hypocalcemia contributes to rickets in global Snx10-deficient mice

Morphological examination of stomachs (n=6) demonstrated global Snx10-deficient mice have a severely pathological appearance of stomach (Figure 3.11 A), suggesting that Snx10 deficiency might result in the GI dysfunction in terms of calcium bioavailability. High expression of SNX10 at mRNA level was found in the the bone as well as in the stomach (Figure 3.11 B, left panel). In the stomach, Snx10 expression was restricted to the body/corpus (Figure 3.11 B, right panel). Immunofluorescence of stomach sections (Figure 3.11 C, upper panel) demonstrated that Snx10 was expressed in gastric zymogenic cells. Zymogenic cells play an essential role in getting soluble calcium from oral food intake for calcium availability to the intestine in aspect of their secretion of digestive enzymes. Immunofluorescence (Figure 3.11 C) and H&E staining (Figure 3.11 D) of stomach sections showed that Snx10 deficiency resulted in much reduced abundance of zymogenic cells. Moreover, global Snx10-deficient mice were found to have the increased gastric pH compared to controls, suggesting gastric acidification was also affected (Figure 3.11 E, left panel, 4.62 +/- 0.43 vs. 2.50 +/-0.32, respectively, n=5 per group, **p<0.01). Taken together, these findings uncovered Snx10 expression in the gastric zymogenic cell and showed that Snx10 deficiency resulted in reduced zymogenic cell abundance and impaired gastric acidification. Because it has been known that impaired gastric acidification affected calcium availability to the intestine (Schinke et al, 2009), we further investigated whether calcium homeostasis was also affected in global Snx10-deficient mice, which could help explain the rachitic aspects of the phenotype. Assessment of serum calcium confirmed that global Snx10-
deficient mice were severely hypocalcemic compared to their controls (6.40 +/- 2.46 mg/dl vs 10.21 +/- 1.81 mg/dl, respectively, n=6 per group, *p< 0.05, Figure 3.11 E, right panel). Moreover, assessment of parathyroid hormone (PTH) showed that global Snx10-deficient mice had a significantly higher PTH level compared with controls (0.142+/-0.02 ng/ml vs 0.75+/-0.13 ng/ml, respectively, n=3 per group, **p<0.01, Figure 3.11F). Taken together, the results suggested that the rachitic phenotype of global Snx10-deficient mice was resulting from impaired calcium homeostasis caused by gastric dysfunction.
Figure 3.11 Gastric dysfunction with subsequent hypocalcemia contributes to rickets in global Snx10-deficient mice

(A) Morphological examination of stomachs showed that global Snx10-deficient mice had a severely pathological appearance of stomach.

(B) qPCR analysis showed that Snx10 was expressed highly at mRNA level in bone as well as in stomach. In the stomach, Snx10 expression was restricted to the body/corpus.

(C) Immunofluorescence of stomach sections demonstrated that Snx10 was expressed in gastric zymogenic cells. Snx10 deficiency resulted in much decreased abundance of zymogenic cells.

(D) Histological analysis confirmed much decreased abundance of zymogenic cells in the stomach of global Snx10-deficient mice.

(E) A significant increase in gastric pH and decrease in serum calcium level of global Snx10-deficient mice compared to controls were detected.

(F) A significant increase in serum parathyroid hormone (PTH) level of global Snx10-deficient mice compared to controls was detected.
Figure 3.11 (continued) Gastric dysfunction with subsequent hypocalcaemia contributes to rickets in global Snx10-deficient mice
Figure 3.11 (continued) Gastric dysfunction with subsequent hypocalcaemia contributes to rickets in global Snx10-deficient mice
To further confirmed this, calcium supplementation was performed to prevent the rachitic phenotype in global Snx10-deficient mice. The diet we used included 2% calcium gluconate and 2,000 IU kg⁻¹ vitamin D, since calcium gluconate have a greater solubility than calcium bicarbonate in the stomach with impaired acidification (Straub, 2007; Schinke et al., 2009). Moreover, due to the tissue damage seen in the stomachs of global Snx10-deficient mice we sought to improve calcium supplementation by also giving sub-cutaneous injections of 2% calcium gluconate diluted in saline. Calcium supplementation was initiated at 14 days after birth and continued for 10 days. Calcium supplementation of global Snx10-deficient mice prevented the premature death seen at 3-4 weeks in the untreated global Snx10-deficient mice. Treatment restored normocalcemia (10.52 +/- 0.43 mg/dl for treated group of global Snx10-deficient vs 10.70 +/- 0.46 mg/dl for the normal control group, n=6, p=0.661) (Figure 3.12 A, left panel). Bone mineral density (BMD) in the treated group of global Snx10-deficient mice was normalized by calcium supplementation (Figure 3.12 A, center panel, n=6, * p<0.05, ** p<0.01). Bone mineral content (BMC) in the treated group of global Snx10-deficient mice was significantly improved, compared to the untreated group of global Snx10-deficient mice (Figure 3.12 A, right panel, n=6, ** p<0.01), however still lower compared to the normal control group (Figure 3.12 A, right panel, n=6, * p<0.05). Radiographic analysis of femur demonstrated that metaphyseal fraying and cupping in the treated group of global Snx10-deficient mice was prevented in contrast to the untreated group of global Snx10-deficient mice (Figure 3.12 B). We next analyzed femur by micro-CT (Figure 3.12 C) and confirmed the amelioration of “moth-eaten” aspect in the treated group of global Snx10-deficient mice. Taken together, these results
suggested that both the mortality and the rickets defect observed in global Snx10-deficient mice were caused by hypocalcaemia and could be prevented by calcium supplementation.
Figure 3.12 The rachitic phenotype in global Snx10-deficient mice was prevented by calcium supplementation

(A) Calcium supplementation restored normocalcemia in the treated group of global Snx10-deficient mice. Bone mineral density (BMD) in the treated group of global Snx10-deficient mice was normalized by calcium supplementation. Bone mineral content (BMC) in the treated group of global Snx10-deficient mice was significantly improved, compared to the untreated group of global Snx10-deficient mice, however still lower compared to the normal control group.

(B) Radiographic analysis of femur demonstrated that metaphyseal fraying and cupping in the treated group of global Snx10-deficient mice was prevented in contrast to the untreated group of global Snx10-deficient mice.

(C) Micro-CT confirmed amelioration of “moth-eaten” aspect in the treated group of global Snx10-deficient mice.
Figure 3.12 (continued) The rachitic phenotype in global Snx10-deficient mice was prevented by calcium supplementation.
Global Snx10-deficient mice died between 3 and 4 weeks post-partum. X-ray of global Snx10-deficient mice revealed not only high radio density showing osteopetrosis as well as metaphyseal fraying /cupping and rachitic widening of the growth plates, indicative of rickets superimposed on osteopetrosis. Global Snx10-deficient mice exhibited excessive bone mass accompanied with enrichment of non-mineralized osteoid, rachitic widening of growth plate and impaired calcium homeostasis, all characteristics of osteopetrorickets (Demirel et al., 2010).

It was thought that osteoclast failure contributed to rickets in osteopetrorickets cases since the majority of calcium resides in skeleton (Kirubakaran et al., 2004; Kaplan et al., 1993). A recent study in mice and humans challenges this notion and puts forward the following provocative hypothesis: osteoclast dysfunction alone cannot be responsible for the imbalance in calcium homeostasis that leads to mineralization defects in rickets (Schinke et al., 2009). The authors demonstrate that rickets is multifactorial due to both impaired gastric acidification and the inability of osteoclasts to resorb bone (Schinke et al., 2009). Our study showed that Snx10 is not only expressed in osteoclast in bone but also expressed in zymogenic cells in stomach. Global Snx10-deficient mice exhibit reduced abundance of zymogenic cells in stomach and increased gastric pH, leading to hypocalcaemia. Global Snx10-deficient mice thus exhibited a complex phenotype that is a combination of osteopetrosis (due to failure) and rickets (due to impaired mineralization and defective calcium homeostasis). This is borne out by the amelioration of the rachitic phenotype when Snx10 is knocked out specifically in
osteoclasts, with normalization gastric acidification, serum calcium level, and bone mineralization. These observations unify the complex phenotype seen in Global Snx10-deficient mice as being due to the simultaneous inhibition of osteoclast function and gastric function.

Furthermore, calcium gluconate supplementation prevented the rachitic phenotype and early death in global Snx10-deficient mice, suggesting that this may be a critical component of the clinical approach to Snx10-dependent human ARO with hypocalcaemia and/or no improvement after HSCT (Sobacchi et al., 2013; Pangrazio et al., 2013).

Our results are in line with those of Schinke et al. (Schinke et al., 2009). Those authors analyzed the combined acid defect phenotype by using mutations in different genes (Cckbr for the stomach and Src for the osteoclasts), whereas our study compares the same gene expressed in both tissues. We found that Snx10 plays an essential role in cross-regulation that exists between stomach and bone. The skeleton, as one of the largest tissues of the vertebrate body plan, does not function in isolation. Instead, cross-talk exists between bone and other organs (Karsenty and Qury, 2012). In response to hypocalcaemia, the stimulated increase in PTH causes more bone resorption in order to mobilize calcium from bone and more renal calcium reabsorption in order to reduce the calcium loss in urine. Meanwhile stimulated vitamin D pathway activates the vitamin D receptor in gut to augment calcium absorption and in bone for more bone resorption. Finally these multistep and multi-organ regulations compensate for low level of serum calcium. In global Snx10-deficient mice, gastric dysfunction led to poor calcium absorption and subsequent hypocalcaemia. PTH level was increased, however bone
resorption was inactivated due to osteoclast failure and calcium absorption in gut was restricted due to gastric dysfunction. In this situation, osteopetrorickets can be expected.

Snx10 was found to appear first in one type of bony fishes, Osteichthyes (http://www.treefam.org/family/TF332117). It was known that the stomach which could secrete acid also evolved in the bony fishes. This “coincidence” is likely to guide us to speculate the possibility that the acidification system in the osteoclasts and the one in the stomach might evolve simultaneously. To date, several molecules involved in the acid producing system were found to be expressed in both cell types: Tcrg-1 (also known as Atp6i), Clc-7 and Ae2, among which, Tcrg-1 (Schinke et al., 2009) and Ae2 (Gawenis et al., 2004; Wu et al., 2008; Josephsen et al., 2009; Schinke et al., 2009) deficient mice display both osteopetrorickets and achlorhydria phenotypes. We demonstrated that Snx10 deficiency resulted in acidification defect both in osteoclasts and stomach. global Snx10-deficient mice exhibited osteopetrorickets. But, Snx10 might not be exactly the same as Atp6i, Clc-7 or Ae2 in aspect of the molecular function(s) since Snx10 deficiency results in not only impaired gastric acidification but also less abundance of zymogenic cells. So it raises another question to be addressed in the future, whether the gastric acidification defect in global Snx10-deficient mice is primary or secondary, since acid secretion (thus, calcium homeostasis) and trafficking of digestive enzymes including pepsinogen C (PGC) are intertwined. It was known that parietal cell acid secretion defect and/or damage to parietal cells causes less mature zymogenic cells abundance (Nam et al., 2010; Bredemeyer, 2009). Therefore, the fate and function of parietal and zymogenic cells are profoundly intertwined (Huh et al., 2012).
The mechanism by which Snx10 regulates the differentiation and/or the function of gastric zymogenic cell will be studied in the future. Our own data in addition to other reports support a concordance between intracellular trafficking in osteoclasts and zymogenic cells. For example, Gnptab-null mutant results in defective intracellular trafficking in both osteoclasts and zymogenic cells (Gelfman et al., 2007; van Meel et al., 2011; Vogel et al., 2009). Gnptab-null mice were also found to have the low BMD (Vogel et al., 2009; Kollmann et al., 2013). Again, this study defined a novel regulatory relationship between the GI tract and the skeletal system. The mouse models used in this study are likely to guide us in the development of a potentially life-saving, simple treatment (calcium supplementation) for osteopetrorickets patients with mutations in SNX10 or functionally related genes. Further, this study will establish novel theme in the interrelationship of basic cellular vesicular transport and acidification mechanisms, calcium bioavailability and mineralization homeostasis in bone health in the general population.
References


CHAPTER 4

Conclusion
Conclusion

It has been known that Snx10 is expressed in osteoclasts and required for osteoclastic bone resorption \textit{in vitro} (Zhu et al., 2012), however the \textit{in vivo} study of Snx10 role in bone biology is lacking. In this study, mouse models in which Snx10 was deficient specifically in osteoclasts was created. Osteoclast-specific Snx10-deficient mice exhibited all characteristics of osteopetrosis, consistent with osteoclast failure due to Snx10 deficiency. Snx10 deficiency did not completely inhibit osteoclast formation, however resulted in reduced resorptive capacity. Osteoclasts from osteoclast-specific Snx10-deficient mice failed to form extracellular acidification. Finally, intracellular vesicle transport and ruffled border formation were found to be impaired in osteoclasts from osteoclast-specific Snx10-deficient mice. To our knowledge, this is the first study to explore the role of Snx10 using a genetically modified mouse model. This study not only underscored the role of Snx10 in osteoclastic bone resorption \textit{in vivo}, but also uncovered the cellular mechanism by which Snx10 regulates osteoclast function.

We also generated global Snx10-deficient mice. The data showed that global Snx10-deficiency in mice results in a combined phenotype: osteopetrosis (due to osteoclast failure) and rickets (due to gastric dysfunction and low calcium availability, resulting in impaired skeletal mineralization). Osteopetrorickets, the paradoxical association of insufficient mineralization in the context of a positive total body calcium balance, was thought to occur due to failure of the osteoclasts to maintain normal calcium homeostasis. However, osteoclast-specific Snx10 deficiency did not affect calcium balance, and therefore resulted in severe osteopetrosis \textit{without} rickets.
Moreover, supplementation with calcium gluconate restored normocalcemia, prevented the rachitic phenotype and rescued the early death in global Snx10-deficient mice, suggesting that this may be a life-saving component of the clinical approach to Snx10-dependent human ARO with hypocalcaemia and/or no improvement after HSCT. The data showed that Snx10 deficiency resulted in a complex phenotype that was a consequence of both deficient osteoclasts and gastric zymogenic cells, uncovering the role of Snx10 in cross-regulation between bone and stomach. It also highlights the interrelationship between zymogenic cell function, gastric function, calcium bioavailability, calcium absorption and their final impact on bone health in the general population. Finally, the results from these studies could change the paradigm of treatment for osteopetrotic patients with mutations in SNX10 and other genes with similar patterns of expression and activities.
References

Supplemental Figures

Supplemental Figure 3.1 X-ray of WT, global Snx10-deficient mice and global Atp6i-deficient mice
Supplemental Figure 3.2 micro-CT 3D reconstruction of surface plane of femur of WT, global Snx10-deficient mice and global Atp6i-deficient mice
Supplemental Figure 3.3 micro-CT 3D reconstruction of surface plane of femur of WT, OC Snx10-deficient mice and global Snx10-deficient mice