TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 906

MEDICA - ODONTOLOGICA

ACID-BASE BALANCE AND OXIDATIVE METABOLISM IN CALCIFIED TISSUES

by

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Turku 2010

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ISBN 978-951-29-4307-4 (PRINT)
ISBN 978-951-29-4308-1 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2010

“If you would be a real seeker after truth, it is necessary that at least once in your life you doubt, as far as possible, all things.”
René Descartes (1596 – 1650)

Riihonen, Riikka: Acid-base balance and oxidative metabolism in calcified tissues.
Institute of Biomedicine, Department of Cell Biology and Anatomy, University of Turku, Finland
Annales Universitatis Turkuensis, Medica-Odontologica, Turku, Finland, 2010

ABSTRACT

The calcified tissues, comprising bone and cartilage, are metabolically active tissues that bind and release calcium, bicarbonate and other substances according to systemic needs. Understanding the regulation of cellular metabolism in bone and cartilage is an important issue, since a link between the metabolism and diseases of these tissues is clear. An essential element in the function of bone-resorbing osteoclasts, namely regulation of bicarbonate transport, has not yet been thoroughly studied. Another example of an important but at the same time fairly unexplored subject of interest in this field is cartilage degeneration, an important determinant for development of osteoarthritis. The link between this and oxidative metabolism has rarely been studied.

In this study, we have investigated the significance of bicarbonate transport in osteoclasts. We found that osteoclasts possess several potential proteins for bicarbonate transport, including carbonic anhydrase IV and XIV, and an electroneutral bicarbonate co-transporter NBCn1. We have also shown that inhibiting the function of these proteins has a significant impact on bone resorption and osteoclast morphology. Furthermore, we have explored oxidative metabolism in chondrocytes and found that carbonic anhydrase III (CA III), a protein linked to the prevention of protein oxidation in muscle cells, is also present in mouse chondrocytes, where its expression correlates with the presence of reactive oxygen species. Thus, our study provides novel information on the regulation of cellular metabolism in calcified tissues.

Keywords: bone, osteoclast, chondrocyte, bicarbonate, carbonic anhydrase

Riihonen, Riikka: Happo-emästasapainon ja oksidatiivisen aineenvaihdunnan säätely kovakudoksessa.
Biolääketieteen laitos, Solubiologia ja anatomia, Turun yliopisto
Annales Universitatis Turkuensis, Medica-Odontologica, Turku, 2010

YHTEENVETO

Luu ja rusto ovat kovakudoksia, joissa tapahtuu jatkuvasti aktiivista aineenvaihduntaa, mikä takaa elimistölle muun muassa kalsiumin, bikarbonaatin ja muiden elektrolyyttien saatavuuden ja varastoimisen tarvittaessa. On tärkeää ymmärtää, miten kovakudoksen solutason aineenvaihduntaa säädellään, koska sitä tuntemalla voidaan saada uudenlainen näkökulma myös luun ja ruston sairauksiin. Esimerkiksi osteoklastien luunhajotukseen olennaisena osana liittyvää bikarbonaatin kuljetusta on tutkittu tähän mennessä vasta hyvin vähän. Toinen tärkeä mutta vielä huonosti tunnettu tutkimusaihe on ruston kuluminen, joka nykykäsityksen mukaan aiheutuu rustosolujen ennenaikaisesta rappeutumisesta. Ruston oksidatiivista aineenvaihduntaa, joka saattaa liittyä tähän rappeutumisprosessiin, ei tunneta juuri lainkaan.

Tässä väitöskirjatyössä selvitettiin osteoklastisolujen bikarbonaatin kuljetusta. Havaitsimme, että osteoklasteissa on useita bikarbonaatin kuljetukseen soveltuvia proteiineja, muun muassa hiilihappoanhydraasit IV ja XIV ja natrium-bikarbonaattivaihtaja NBCn1. Näiden proteiinien toiminnan estäminen johti merkittäviin muutoksiin luun hajotusnopeudessa ja osteoklastien solumorfologiassa. Tutkimme myös oksidatiivista aineenvaihduntaa rustossa tarkastelemalla hiilihappoanhydraasi III:n (CA III) ilmenemistä eri-ikäisten hiirten rustokudoksessa. CA III, joka lihassoluissa toimii suojaavana tekijänä estäen proteiinien hapettumista, esiintyy havaintojemme mukaan hiiren rustossa proteiinitasolla samoilla alueilla kuin reaktiiviset happiradikaalit, joita kyseisessä kudoksessa ilmenee. Näin ollen tutkimuksemme on tuottanut uutta tietoa kovakudoksen aineenvaihdunnan säätelystä.

Avainsanat: luu, osteoklasti, kondrosyytti, bikarbonaatti, hiilihappoanhydraasi

CONTENTS

ABBREVIATIONS

$1,25(OH)_2D_3$	1,25-Dihydroxyvitamin D3
AE	Anion exchanger
ALP	Alkaline phosphatase
ARNT	Aryl hydrocarbon receptor nuclear translocator
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CA	Carbonic anhydrase
CD14	Cluster of differentiation 14
ClC7	Chloride channel 7
CO_2	Carbon dioxide
CSF	Colony-stimulating factor
CT	Calcitonin
DIDS	4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid
DIPH	Diphenhydramine
ER	Endoplasmic reticulum
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
FGF	Fibroblast growth factor
FSD	Functional secretory domain of osteoclast membrane
GSH	Glutathione
GSSG	Glutathione disulfide
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIF	Hypoxia-inducible factor
IGF-1	Insulin-like growth factor 1
Ihh	Indian hedgehog
IL	Interleukin
KCC1	Potassium/chloride co-transporter 1
M-CSF	Macrophage colony-stimulating factor
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NBCe1	Electrogenic bicarbonate/sodium exchanger 1
NBCn1	Electroneutral Na^+-dependent bicarbonate/sodium exchanger
NH^{4+}	Ammonium ion
NHE	Sodium/proton exchanger protein
OP-1	Refers to bone morphogenetic protein 7
OPG	Osteoprotegerin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
pH_e	Extracellular pH
pH_i	Intracellular pH
PO_4	Phosphate

PTH	Parathyroid hormone
pVHL	von Hippel-Lindau protein
RANK	Receptor activator for nuclear factor κB
RANKL	Ligand for receptor activator for nuclear factor κB
ROS	Reactive oxygen species
RUNX2	Osteoblast-specific transcription factor
shRNA	Small hairpin ribonucleic acid
SLC4A	Solute carrier family 4
Sox9	SRY (sex determining region Y)-box 9
TAL	Thick ascending limb in the kidney
TGFβ	Transforming growth factor β
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAcP	Tartrate-resistant acid phosphatase
TRAF	Tumor necrosis factor receptor associated factor
V-ATPase	Vacuolar-type proton adenosine triphosphatase
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by Roman numerals I-III. In addition, unpublished results are included.

I Riihonen R, Supuran CT, Parkkila S, Pastorekova S, Väänänen HK, Laitala-Leinonen T. Membrane-Bound Carbonic Anhydrases in Osteoclasts. Bone 2007 40(4):1021-1031.

II Riihonen R, Nielsen S, Väänänen HK, Laitala-Leinonen T, Kwon T-H. Degradation of hydroxyapatite *in vivo* and *in vitro* requires osteoclastic sodium-bicarbonate co-transporter NBCn1. Matrix Biology 2010 29(4): 287-294.

III Riihonen R, Heinonen J, Säämänen A-M, Laitala-Leinonen T. Carbonic anhydrase III – a regulator of embryonal chondrogenesis. *Submitted.*

1 INTRODUCTION

Bone is a fascinating tissue with multiple functions, all crucial to the well-being of an individual. First of all, bone protects fragile inner organs from mechanical damage. Secondly, bone carries the weight of the body, constantly remodeling itself to respond to mechanical loading. Thirdly, bone stores and supplies multiple minerals, mostly calcium and phosphate, the amounts of which in plasma are tightly regulated at the systemic level. Fourthly, bone acts as an endocrine organ, secreting hormones, and fifthly, bone actively buffers changes in the systemic pH.

Bone turnover rate is largely defined by the rate of osteoclastic bone resorption. Thus, understanding molecular mechanisms for bone resorption may provide a key to the pathophysiology of diseases related to excess osteoclastic activity such as osteoporosis. Osteoclasts resorb bone by creating a closed space between their cell membrane and the bone surface, where they excrete acid to dissolve minerals from the organic bone matrix. Therefore, osteoclasts possess a variety of proteins for proton production and transport, both essential processes for the acidification of the resorption lacuna. As minerals are dissolved at the resorption site from the bone matrix, great amounts of carbonate and bicarbonate are released as well. The released bicarbonate would rapidly alkalize the resorption pit, ceasing bone resorption if it remained in the resorption lacuna. Thus, bicarbonate transport mechanisms are necessary in functional osteoclasts for effective bone resorption.

The properties of chondrocytes, in turn, are major determinants for fetal bone formation and postpartum bone growth. Chondrocyte proliferation and maturation are tightly regulated processes, and during endochondral ossification these processes eventually lead to the replacement of hypertrophic cartilage with newly formed bone. Cellular metabolism of chondrocytes has been shown to be highly dependent on environmental factors such as hypoxia, which primes mesenchymal stem cells to become chondrocytes and regulates the expression of cartilage-specific proteins. Furthermore, reactive oxygen species (ROS) arising due to alterations in cartilaginous oxygen levels during chondrocyte maturation have been found to impinge on chondrocyte function. The mechanisms of how chondrocytes survive and escape harmful effects of hypoxia and ROS have remained mostly unclear, however.

This study concentrated on exploring two aspects of the role of carbonic anhydrases in metabolism in calcified tissues: bicarbonate transport in osteoclasts and oxidative metabolism in chondrocytes. The study provides novel information on the regulation of cellular metabolism in these tissues.

2 REVIEW OF THE LITERATURE

2.1 Introduction to bone and cartilage

2.1.1 Bone structure and bone cells

Bone can be divided into two types: cortical (compact) and trabecular (cancellous) bone. Cortical bone is located on the surfaces of bones where it lines the inner trabecular bone to provide mechanical support. It has a well-organized structure with bone tissue arranged in concentric lamellae to surround channels for veins and blood vessels, known as Haversian canals. Trabecular bone is mostly located in the epiphyseal plates of long bones and in the inner parts of smaller bones. It consists of thin trabeculae which serve as a metabolic reservoir in bone turnover but also contribute to the weight-carrying function of bones.

Bone matrix mainly consists of extracellular calcified matrix composed of collagen type I and inorganic minerals. Very few other collagen forms than type I are present in bone, this being a special feature of bone compared to other connective tissues. Only 10-15% of the total protein of bone is non-collagenous, but this lot has an important dual function: it binds bone minerals to the matrix and makes minerals more soluble at the bone surface (Menanteau et al. 1982; Neuman et al. 1982). Minerals account for approximately 70% of adult bone weight, predominantly comprising calcium and phosphorus in the form of hydroxyapatite crystals. The crystals are mainly plate-shaped and very small, thereby providing best protection from fracture (Eppell et al. 2001). Since apatite crystals are formed in the presence of CO_2 in the human body, also remarkable amounts of carbonate become incorporated into the crystals, making the crystals smaller and more soluble. Moreover, heavy metal impurities such as lead, cadmium and strontium may be incorporated in apatite crystals instead of calcium to form even smaller crystals (Robey and Boskey 2003). Magnesium, a dietary cation, and bicarbonate are located on the crystal surface.

Bone contains four types of metabolically active cells. Osteoblasts are bone-forming cells which differentiate from mesenchymal stem cells (MSCs) when a distinct set of transcription factors is present. Especially transcription factors RUNX2 and Osterix are known to be necessary for osteoblastic differentiation (Lian et al. 2004). Osteoblast maturation requires another panel of growth factors, such as Transforming growth factor β (TGFβ), fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) (Ducy et al. 2000b; Schinke and Karsenty 1999). Osteoblasts are mostly located at bone surfaces where bone formation is possible, i.e. at sites where bone resorption has recently occurred. Attracted to these sites by chemotaxis mediated by components of bone (Mundy and Poser 1983), osteoblast precursors differentiate at bone surfaces and start to secrete collagen type I, followed by non-collagenous proteins. The result of this cascade is the appearance of osteoid, unmineralized bone matrix structures. Functional osteoblasts start to express alkaline phosphatase (ALP), an important biochemical marker for osteoblasts. Mineralization of the osteoid is

initiated as matrix vesicles containing calcium and phosphate bud from the osteoblast cell membrane and release their content to specific sites in a process called primary mineralization (Morris et al. 1983). As mineral crystals have formed, secondary mineralization takes place causing crystal growth and thereby further increasing bone strength. Additionally to their bone-forming function, osteoblasts are important regulators of osteoclast differentiation and activity, since they are an essential source of growth factors such as Macrophage colony-stimulating factor (M-CSF) and osteoprotegerin (OPG). They also contain receptors for Receptor Activator for Nuclear Factor κ B Ligand (RANKL), parathyroid hormone (PTH) and vitamin D which are important regulators for bone remodeling.

Osteocytes are the most numerous cells in bone. They lie embedded in individual lacunae inside the bone matrix, having led scientists for a long time to believe they are inactive cells. On the contrary, osteocytes are communicative and active cells which sense mechanical loading and deliver information forward to other bone cell types and even secrete hormones (Bonewald 2007). Osteocytes form a vast network connecting each cell with long, thin processes which pass through the bone matrix and contain gap junctions allowing continuous cell-cell communication (Gu et al. 2006). Information is thought to be delivered from one osteocyte to another in multiple ways, for example by releasing calcium, prostaglandins and nitric oxide through their cellular processes (Ajubi et al. 1996; Fox et al. 1996; Klein-Nulend et al. 1995) or in the form of extracellular fluid flow caused by mechanical forces. The exact mechanisms of osteocyte function have yet remained somewhat unclear but increasing evidence suggests that osteocytes act as primary monitors for the need of initiating bone remodeling in case microfractures occur or if the level of mechanical stress increases (Skerry et al. 1989, Hazenberg et al. 2009). The importance of this probable role of osteocytes can be highlighted by observations indicating that antiosteoporotic medication may partly work via inhibition of osteocyte apoptosis (Boyce et al. 2002; Plotkin et al. 2005). In addition, osteocytes are though to support their environment by laying matrix molecules into their surroundings. Recent studies indicate that the most intriguing function of osteocytes might be related to systemic endocrinology. Osteocytes have been observed to be the main source of FGF-23 in bone, a hormone which regulates phosphate metabolism and vitamin D levels in serum (Liu et al. 2006; Ubaidus et al. 2009; Yoshiko et al. 2007).

Bone lining cells, members of the osteoblast lineage, are located on bone trabeculi when they are not being remodeled, thus functioning as a membranous covering layer for bone tissue. The specific features of these cells as well as their role are quite poorly known, although more and more scientists are becoming inspired by them. Bone lining cells contain very few cell organelles, have flat or slightly ovoid nuclei and connect to each other via gap junctions (Miller et al. 1989). Apparently, these cells can be induced to proliferate and differentiate into osteogenic cells. Osteoblasts and bone lining cells also seem to be in close contact with each other and sometimes joined together with adherent junctions. Moreover, some studies have suggested that bone lining cells may interact with osteocytes via their processes to the surface canaliculi (Islam et al. 1990). Bone lining cells express RANKL and intercellular adhesion molecule 1 (ICAM-1)

(Tanaka et al. 2000), thereby being able to stimulate osteoclast precursors. They might also function in cleaning bone surface through matrix metalloproteinase (MMP) activity prior to osteoclastic bone resorption.

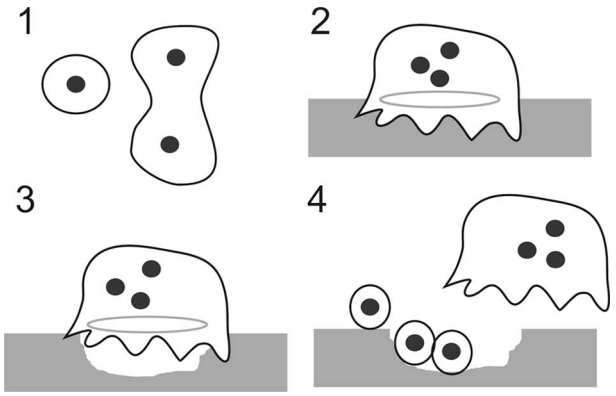


Figure 1:
Phases of bone resorption (for review, see Väänänen and Laitala-Leinonen 2008). 1) Fusion of mononuclear precursor cells to multinuclear osteoclasts 2) Attachment of osteoclasts to bone 3) Bone resorption by acidification of the resorption lacuna and subsequent secretion of proteinases 4) Detachment of osteoclasts from bone and infiltration of the resorption pit by osteoblasts.

Osteoclasts are specialized bone-resorbing cells of hematopoietic origin (for review, see Väänänen and Laitala-Leinonen 2008). They have several distinct features separating them from other bone cells, including multiple nuclei, numerous mitochondria for efficient energy production and the ability to move. Interestingly, they are apparently an end point for one differentiation pathway in the monocyte-macrophage lineage, and since they do not divide, it has not been possible to produce a pure osteoclast cell-line. Osteoclasts resemble macrophages and dendritic cells to some extent, sharing similar cell machinery such as multiple lysosomes. Osteoclasts are also thought to utilize the antigen-presenting feature typical for T, B and NK cells in the monocyte-macrophage lineage, but the significance of this action remains unknown. Moreover, recent data suggests that osteoclasts can form from mature dendritic cells through a process called transdifferentiation (Alnaeeli and Teng 2009; Speziani et al. 2007).

2.1.2 Bone resorption

Mononuclear osteoclast precursor cells circulate in blood and invade bone after sensing homing signals from mesenchymal stromal cells expressing cytokines and growth factors. Especially growth factors RANKL and M-CSF are essential for the homing, differentiation and function of osteoclasts, and osteoclast precursors express receptors for both of these agents (Takahashi et al. 2002; Wong et al. 1997; Yasuda et al. 1998b). After homing and fusion into mature multinuclear osteoclasts, the cells attach to bone surface with the help of integrins. Simultaneously, actin released from intracellular podosomes is converted into filamentous actin, and these two types of actin form a dense ring-like structure near to the bone-facing plasma membrane that interacts with proteins of the extracellular matrix via integrins (Väänänen and Horton 1995). While the actin ring forms, another distinct feature of osteoclasts becomes visible: the osteoclast cell membrane becomes polarized and divides into specific domains as the

cell prepares for its functional activity (Lakkakorpi and Väänänen 1996; Mulari et al. 2003; Palokangas et al. 1997; Salo et al. 1996). A sealing zone is formed at the site where filamentous actin interacts with the bone surface. Inside this area, ruffled border becomes the main locus for ion and protein transport to and from the resorption lacuna. The osteoclast basolateral membrane (non-bone facing plasma membrane) contains ion transporters and channels that are important in maintaining the intracellular ion and pH homeostasis (Väänänen and Laitala 2008). The functional secretory domain (FSD) is located at the top of the osteoclast and it facilitates the transcytosis of bone resorption debris (Salo et al. 1997).

After osteoclasts have adhered to bone, the osteoclastic bone resorption cycle begins with dissolution of inorganic minerals from bone, continues with resorption of the organic bone matrix, and the cycle ends with disposal of waste products. In order to perform these phases, osteoclasts need a continuous supply of energy, a reasonable ion flux and intensive vesicular trafficking. The first step towards mineral dissolution occurs when the ruffled border is formed. A large number of intracellular acidic vesicles fuse to the bone-facing plasma membrane and release acid into the space formed between bone surface and the cell membrane, namely the resorption pit. This results in a pH drop in the space, allowing mineral dissolution to be initialized. Simultaneously, vacuolar proton ATPase (V-ATPase) -containing vesicles fuse into the ruffled border and the V-ATPases continue pumping protons across the cell membrane in order to maintain intralacunar pH levels at 4.5–4.8 (Baron et al. 1985; Fallon 1984; Väänänen et al. 1990). A chloride channel ClC7 at the ruffled border enables the formation of hydrochloric acid at the resorption site (Kornak et al. 2001). Protons for the action of V-ATPases are continuously formed in osteoclasts in a cytoplasmic reaction facilitated by carbonic anhydrase II (CA II), where carbon dioxide and water are converted into bicarbonate and protons (Sundquist et al. 1987). Furthermore, osteoclasts secrete lysosomal proteases which allow the degradation of collagen and other organic components of bone: cathepsins (mainly cathepsin K), matrix metalloproteinases such as MMP-9 and MMP-14, and tartrate-resistant acid phosphatase (TRAcP). Thereafter, bone degradation products are endocytosed and transported through the cell. This transcytosis requires major vesicular trafficking across the osteoclast (Salo et al. 1997), but ions are probably also taken into osteoclasts via specific channels and transporters, such as the calcium channel. Degradation of the endocytosed organic material continues during vesicular trafficking with the help of TRAcP facilitating reactive oxygen species (ROS) production in the vesicles for this purpose. After completing bone resorption, osteoclasts detach from bone and, *in vitro*, either die via apoptosis or move to start resorption at another site. *In vivo*, the fate of individual osteoclasts after resorption is not known, although the cells will eventually go into apoptosis. After resorption, the lacuna is occupied by mononuclear, osteoblast-like cells that clean up remains of the resorbed bone matrix before bone formation begins. During this phase, bone surface becomes covered with an osteopontin-rich layer that is thought to act as connective material between new and old bone.

2.1.3 Chondrocytes and the structure of the growth plate

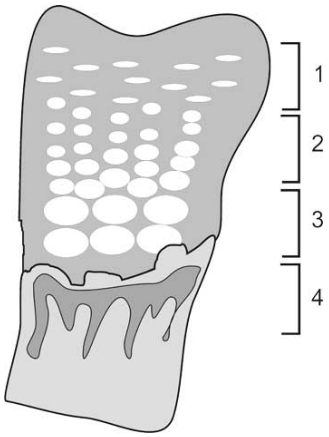


Figure 2: Long bone growth takes place at the cartilaginous growth plate. Chondrocytes at the 1) resting zone 2) proliferative zone 3) and hypertrophic zone. 4) The calcified cartilage is replaced by trabecular bone.

Cartilage is tough tissue occupying the epiphyses and articular surfaces of long bones as well as the nose, auricle, trachea and bronchi in an adult human. Three types of cartilage are found in the body: hyaline cartilage, fibrocartilage and elastic cartilage, the features of which are unique to each type. Hyaline cartilage is the most common cartilage type, occupying the surfaces of long bones in adults and also contributing to embryonal endochondral ossification. Cartilage contains only one cell type, the chondrocyte, which arises from the mesenchyme during embryonal chondrogenesis. Along chondrogenesis, mesenchymal stem cells first form prechondrogenic condensations at the prospective skeletal sites. Thereafter, the fibroblast-like shape of these MSCs transforms to the typical spherical morphology of chondrocytes and the synthesis of cartilage-specific matrix molecules such as type II, IX, and XI collagen and aggrecan begins (for review, see Bobick et al. 2009). Cartilage is remodeled by chondroclasts (septoclasts) although at present there is only limited knowledge about the possible similarities or differences between osteoclasts and chondroclasts. The mechanisms of cartilage resorption hence remain largely unknown.

Bone formation occurs via two types of processes: intramembranous and endochondral ossification. Intramembranous ossification refers to a process where bone is directly formed inside the mesenchyme. This process mostly occurs in the facial region and the cranial vault, resulting in the formation of flat bones. Formation of long bones, in turn, occurs via endochondral ossification in a controlled two-step process, where a cartilagenous anlage is first formed and then remodeled into bone (Alini et al. 1996). While chondrocytes proceed to hypertrophy and die, cartilage matrix becomes calcified. Matrix degrading enzymes such as MMP-2, MMP-9, MMP-10, and MMP-13 promote matrix degradation and make room for blood vessel sprouting which is invited to the site by Vascular endothelial growth factor (VEGF) secreted by hypertrophic chondrocytes (Blumer et al. 2008; Gerber et al. 1999). Bone formation by osteoblasts spreads along the cartilage template until the front of the bone is approached. Thereafter, distal chondrogenesis elaborates the growth plate, allowing longitudinal bone growth during childhood and puberty.

In the growth plates (GPs) of long bones, chondrocytes are arranged in three layers according to their status: the resting, proliferative and hypertrophic zones (for review see Mackie et. al 2008). In the resting zone, chondrocytes are irregularly arranged in a bed of cartilage matrix and rarely divide, acting as stem cells that replenish the pool of proliferative chondrocytes (Abad et al. 2002). They secrete anti-angiogenic factors and synthesize a matrix that mainly consists of type II collagen and proteoglycans. Farther toward the metaphysis in the proliferative zone, chondrocytes are arranged in columns oriented parallel to the long axis of bone. During the proliferative/prehypertrophic stage, bone morphogenetic protein-6 (BMP-6), adseverin, RUNX2, Osterix and Indian hedgehog (Ihh) are produced (Mackie et al. 2008; Zuscik et al. 2008). The proliferative chondrocytes farthest from the epiphysis stop replicating and enlarge to become hypertrophic. During this process, chondrocyte size increases 6- to 10-fold, thus contributing to longitudinal bone growth (Hunziker 1994). Chondrocytes start to express collagen X and osteogenic genes, such as alkaline phosphatase and osteonectin (for review, see Gerstenfeld et al. 1996). The matrix around hypertrophic chondrocytes is mineralized through deposition of hydroxyapatite. The width of the growth plate remains relatively constant while new bone is formed at the chondro-osseus junction. Chondrogenesis becomes slower with age, and there are theories suggesting that this decline occurs because the cells in the resting zone have a finite proliferative capacity that is gradually exhausted. In humans, this exhaustion is thought to occur during puberty as epiphyseal fusion terminates longitudinal growth.

Chondrocytes are regulated by several local and peripheral growth factors, such as BMPs exerting articular cartilage development and joint formation. BMPs are also used to support chondrogenic differentiation of mesenchymal stem cells *in vitro* (Oshin and Stewart 2007). BMP-2 is expressed in areas surrounding the initial cartilage condensations, while BMP-4 is expressed in the perichondrium. BMP-6 is expressed in hypertrophic chondrocytes, whereas high levels of BMP-7 mRNA have been observed in the perichondrium (Zhao et al. 2002). BMP-7, also known as OP-1, has been shown to play a unique role in cartilage: it induces matrix synthesis and is used to repair cartilage *in vivo* in various models of articular cartilage degradation, including focal osteochondral and chondral defects and osteoarthritis (Chubinskaya et al. 2007). BMP-7 also stimulates the formation of cartilage-specific extracellular proteins in chondrocytes, including type II and VI collagen, aggrecan, decorin, fibronectin and hyaluronan (Grgic et al. 1997). Moreover, BMP-7 modulates the expression of other growth factors in cartilage, such as the Insulin-like growth factor-1 (IGF-1), other BMPs and the interleukin 6 (IL-6) family of proinflammatory cytokines (Chubinskaya et al. 2007; Im et al. 2003). It also possesses anti-catabolic properties that may be of high importance in the regulation of cartilage cell survival. Furthermore, chondrocyte proliferation is regulated by interaction with parathyroid hormone / parathyroid hormone related peptide (PTH/PTHrP), Ihh and Transforming growth factor β (TGF-β) in a negative feedback loop mechanism (Alvarez et al. 2002; Vortkamp et al. 1996). The PTH/PTHrP system maintains chondrocyte proliferation and inhibits hypertrophy, whereas Ihh promotes chondrocyte hypertrophy through its action on TGFβ. The synthesis of Ihh is increased, in turn, by RUNX2 transcription factor which also

increases osteoblast differentiation and matrix production (Yoshida and Komori 2005). In addition, IGF-1 localized in upper hypertrophic chondrocytes in the GP cartilage plays a role in chondrocyte maturation (Reinecke et al. 2000).

2.2 Regulation of bone metabolism by environmental factors

Continuous bone formation and resorption serve an important metabolic function, aiming at the release or binding of substances such as calcium, phosphate and physiological buffers such as bicarbonate according to systemic needs. Understanding the complex network of environmental factors that affect bone cell metabolism is needed when using *in vitro* cell cultures which lack physiological cell-cell interactions and hormonal/neuronal stimuli.

2.2.1 Endocrine regulation in bone

Parathyroid hormone is secreted from the parathyroid glands when the parathyroid hormone plasma membrane Ca-sensing receptor (CaSR) detects a decrease in serum calcium. Secretion of PTH results in enhanced renal tubular reabsorption of Ca^{2+}, increased osteoclastic bone resorption and stimulation of intestinal calcium absorption indirectly through increased 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) synthesis. PTH is also a major phosphaturic factor that stimulates the excretion of phosphate by the kidneys. At the bone cell level, PTH functions via the osteoblast lineage where the PTH receptor is expressed, and it has no direct impact on osteoclasts (Shinoda et al. 2010, Takahashi et al. 2002). PTH stimulates the expression of RANKL in osteoblasts, decreases OPG expression and increases the synthesis of M-CSF, hence resulting in indirect osteoclast activation (Shinoda et al. 2010). Interestingly, PTH increases the number of osteoblasts through various mechanisms but also inhibits the maturation of preosteoblasts and decreases the production of collagen and other matrix proteins by mature osteoblasts (Aubin and Triffit 2002; Dobnig and Turner 1995; Manolagas 2000). There is evidence that the net effect of PTH on bone is site-specific and may be anabolic in long bones (Kishi et al. 1998).

Vitamin D plays a major role in the regulation of mineral metabolism. Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) can both be utilized in hydroxylation reactions in humans, resulting in the biologically active form ($1,25(OH)_2D_3$). The main function of vitamin D is to maintain adequate serum calcium levels in order to avoid hypocalcemia. This is achieved by increasing intestinal absorption of calcium via vitamin D receptor in the small intestine. $1,25(OH)_2D_3$ also enhances the absorption of phosphorus from the gastrointestinal tract. Furthermore, especially in case of inadequate calcium intake, vitamin D interacts with its receptor in osteoblasts to increase RANKL expression, leading to osteoclast activation and increased bone resorption. It also increases the expression of ALP, osteocalcin, osteonectin and OPG by osteoblasts (for review, see St-Arnaud 2008).

Calcitonin (CT) is a peptide hormone secreted by the thyroid parafollicular C cells, that inhibits bone resorption by rapidly decreasing osteoclast size and activity, and it is therefore clinically used as an antiresorptive therapy. The blood calcium level has a direct impact on calcitonin secretion via CaSR but also gender and age affect the basal

levels of calcitonin. Calcitonin has been observed to inhibit osteoclastic proton extrusion through protein kinase A activation (Kajiya et al. 2003). Moreover, calcitonin has been shown to inhibit ostecyte apoptosis (Plotkin et al. 1999). Interesting observations about high basal calcitonin levels in pycnodysostosis, a rare dysfunction of the cathepsin K gene, suggest that calcitonin has a significant impact on bone remodeling (Baker et al. 1973) but exact mechanisms are not known. Recent findings indicate that calcitonin may be a neurotransmitter and its receptors are present in the central nervous system (Hosokawa et al. 2010).

Estrogen (representing estradiol (E2) and estrone) is a major sex steroid hormone in women. The reduction of systemic estrogen secretion in postmenopausal women leads to pathological bone loss, and estrogen replacement therapy is an effective method to prevent and treat postmenopausal osteoporosis (Härkönen and Väänänen 2006). A certain level of estrogens is also needed in men to ensure bone health (for review, see Vandenput and Ohlsson 2009). Estrogen signals through two receptors, ERα and ERβ (Kuiper et al. 1996), but ERα mediates most actions of estrogen on bone. Osteoblasts, osteocytes and osteoclasts express both ERα and ERβ (Komm et al. 1988; Oursler et al. 1991; Tomkinson et al. 1998). Estrogen has been observed to promote a lineage shift toward osteoblasts in the MSC differentiation process *in vitro* and to prevent osteoblast apoptosis, thereby providing bone with protective signals. By contrast, estrogen inhibits osteoclastogenesis by reducing local production of regulative cytokines and increases osteoclast apoptosis, a phenomenon recently linked to the ERβ receptor (Cruzoé-Souza et al. 2009). A study in an *in vitro* bone resorption assay indicated that organic matrix degradation by osteoclasts was disturbed because of exposure to E2 (Parikka et al. 2001). There are only few pieces of evidence about any effects of progesterone, the other major female sex hormone, on bone.

Androgens have profound influence on bone in both male and female individuals, and decreased androgen levels have been linked to reduced bone mineral density (BMD) in both sexes. Most of the effects of androgens on bone seem to be mediated via osteoblasts which express the androgen receptor mRNA (Abu et al. 1997). Androgens have been detected to stimulate the proliferation of cultured osteoblasts (Kasperk et al. 1990; Kasperk et al. 1989) but the effects of androgens on osteoblast differentiation are not clear at present. Androgen receptors have been detected in avian, mouse, rat and human osteoclasts (Kasperk et al. 1997, Mizuno et al. 1994; Pederson et al. 1999). Osteoclastic bone resorption is thought to be inhibited due to androgens both directly via specific androgen receptors on the osteoclast cell surface and indirectly via osteoblasts and the RANK-RANKL system (Liu et al. 2007; for review, see Vanderschueren and Bouillon 1995). Androgen replacement therapy has been shown to be effective in the treatment of osteoporosis in both male and female patients, although E2 seems to be more effective in this regard (Khosla et al. 2002).

Leptin is a hormone produced mainly by adipose tissue (Considine et al. 1996). Bone marrow stromal cells, GP chondrocytes (Reseland et al. 2001) and the murine fetal osteocartilaginous template (Hoggard et al. 1997) have been shown to express leptin receptors. The net impact of leptin on bone is considered to be dual. Leptin may

enhance estrogenic effects on bone and contribute directly to the differentiation of stromal cells between osteoblast and adipocyte pathways (Reseland et al. 2001). In addition, as a paracrine hormone leptin may decrease bone resorption by modulating the OPG-RANKL ratio (Burguera et al. 2001; Gabay et al. 2001). Further studies have shown another functional signaling route for leptin that favors bone resorption, mediated through hypothalamic neurons and the sympathetic nervous system (Karsenty 2006). The main signaling pathway from leptin to bone may involve brain-derived serotonin, which as such favors bone formation, and the synthesis of which is inhibited by leptin (Yadav 2009).

Cortisol is a glucocorticoid hormone secreted by the adrenal cortex. It is a stress hormone essential for the survival of an individual, and glucocorticoids (GCs) are widely used as anti-inflammatory medication in diseases such as asthma and inflammatory bowel disease. However, continuous use of glucocorticoids has serious effects on bone mineral density. Currently, glucocorticoid-induced osteoporosis is the most common type of osteoporosis in adults aged 20–45 years and the most common type of iatrogenic osteoporosis (Khosla et al. 1994). Glucocorticoids interact with osteoblastic glucocorticoid receptors, via which most of the effect of GCs on bone are thought to be mediated. Most studies suggest that GCs inhibit osteoblast function by decreasing cell proliferation and terminal differentiation, and by promoting apoptosis of osteoblasts and mature osteocytes (O'Brien et al. 2004; Weinstein et al. 1998). GCs have, in addition, been shown to have an impact on the synthesis and release of IGFs in osteoblasts, leading to decreased type I collagen synthesis and decreased bone formation (Rosen et al. 2004). Mature osteoclasts lack glucocorticoid receptors (Beavan et al. 2001), and the increased resorption during GC treatment is thought to occur via indirect effects on the RANKL/OPG system, resulting in increased osteoclastogenesis (Hofbauer et al. 1999). Indeed, GCs prime bone marrow-derived monocytes/macrophages to differentiate into mature osteoclasts *in vitro* (Takuma et al. 2003). At the systemic level, GCs decrease intestinal absorption of calcium and increase urinary excretion of phosphate and calcium, which may also contribute to overall bone loss due to GC treatment.

2.2.2 Neuronal regulation in bone

Bone is strongly innervated (Calvo 1968, Mach et al. 2002) and both osteoclasts and osteoblasts possess beta2-adrenergic receptors (β2AR), exposing them to regulation by postsynaptic adrenergic stimulation (Arai et al. 2003, Takeda et al. 2002). Mice and rats treated with the non-selective beta-adrenergic blocker propranolol have an increased bone mass, whereas mice treated with selective agonists or the non-selective beta-agonist isoproterenol exhibit a low bone mass (Bonnet et al. 2005, Takeda et al. 2002). Moreover, genetic disruption of the *β2AR* gene leads to high bone mass in the absence of other abnormalities in hormonal or metabolic status (Elefteriou et al. 2005). β2AR-deficient mice also possess abnormalities in osteoclastogenesis, but this is thought to result from an indirect inhibition of RANKL expression by osteoblasts, rather than a direct impact on osteoclast precursors. In humans, chronic sympathetic activation is associated with low BMD (Yirmiya R at al. 2006), and beta-blockers decrease the overall fracture risk in many but not all studies in this field (Schlienger et

al. 2004). Furthermore, endocannabinoids such as anandamine and 2-arachodonoylglycerol and their cannabinoid receptors CB1 and CB2 as well as transient receptor potential vanilloid type 1 (TRPV1) are present in human osteoclasts (Rossi et al. 2009). Apparently, they modulate the adrenergic signaling in bone, possibly by affecting the release of noradrenalin from postsynaptic nerve terminals (Ishac et al. 1996).

Further addressing the regulative role of the nervous system in bone, several other neuropeptides have also been detected in bone tissue. For instance, glutamate sensitive nerve fibers and N-methyl D-aspartate (NMDA) receptors for glutamate have been isolated in bone (Chenu 2002, Serre et al. 1999). Moreover, serotonin may appear to be a major regulator of bone metabolism (Gustafsson et al. 2006), and dopamine transporter-deficient mice have been shown to display a low bone mass phenotype, linking dopamine signaling to the regulation of bone homeostasis (Bliziotes et al. 2000). Another intriguing neurotransmitter present in bone is calcitonin gene-related peptide (CGRP) which is richly distributed in sensory neurons innervating the skeleton. Studies indicate that CGRP signaling maintains bone mass by directly stimulating stromal cell osteoblastic differentiation and by inhibiting RANKL-induced osteoclastogenesis and bone resorption (Wang et al. 2009).

2.2.3 Relation of bone metabolism to blood calcium and phosphorus

Bone cell function is directly regulated by serum calcium levels in addition to indirect calcium-related hormonal control. In osteoclasts, acidification of the resorption lacuna releases massive amounts of calcium into the resorption space (Silver et al. 1988). Osteoclasts express calcium sensing receptors such as CaSR, calcium channels and the ryanodine receptor, which has led scientists to hypothesize that osteoclasts may be directly regulated by the calcium they release (Bennett et al. 2001; Marie 2009; Ritchie et al. 1994; Zaidi et al. 1995). Indeed, there are *in vitro* observations suggesting that high extracellular Ca^{2+} may directly inhibit bone resorption (Datta et al. 1989; Miyauchi et al. 1990), but there is also data showing that the sensitivity of osteoclasts to inhibition by calcium is dependent on their activation phase, and resorbing osteoclasts are quite resistant to regulation by calcium (Lakkakorpi et al. 1996). External Ca^{2+} is thought to directly affect ion transport in osteoclasts, such as activation of Cl^- channels (Sakai et al. 1999; Shibata et al. 1997) and inhibition of K^+ channels (Arkett et al. 1994; Hammerland et al. 1994), and Ca^{2+} and Mg^{2+} have been observed to be capable of inhibiting V-ATPases (Sakai et al. 2006). On the other hand, removal of calcium may induce osteoclastic bone resorption due to an increase in the number of osteoclasts, which may be related to enhanced osteoclast survival (Iwamoto et al. 2004). This enhanced survival is also thought to explain the increased numbers of osteoclasts found in some osteopetrotic mutations in humans (Nielsen et al. 2007). Regarding osteoblasts, some studies suggest that CaSR is solely responsible for direct sensing of calcium (Kanatani et al. 1999; Marie 2009), while other studies provide evidence for additional major calcium-sensing receptors in osteoblasts, such as ObCASR, and possible redundant mechanisms (Pi and Quarles 2004). Curiously, there is a study showing that in a culture of human monocytes exposed to elevated

extracellular calcium concentrations, monocyte-conditioned stimulation of osteoblast proliferation is amplified (Kanatani et al. 1991).

The adult human body contains approximately 600–700 g of phosphorus mainly in the form of phosphate (PO_4), approximately 85% of which is present in bone (Favus et al. 2006). Unlike calcium, serum PO_4 levels fluctuate widely depending on sex, age, dietary intake, rate of growth, and levels of several hormones. At the individual level, the amount of phosphate in serum is largely determined by the efficiency of PO_4 reabsorption by the kidneys. Regulation of PO_4 levels also includes alterations in the absorption of dietary phosphorus in the small intestine and mobilization of PO_4 from bone. Dietary absorption of phosphorus is dependent on both passive and active transport. $1,25(OH)_2D_3$-dependent active transport in the gastrointestinal tract is of importance particularly in chronic renal failure and vitamin D deficiency, where absorption of PO_4 is increased via this mechanism (Favus et al. 2006). As for renal regulation, a low intake of PO_4 stimulates reabsorption in the kidneys, and vice versa, and these changes are independent of the extracellular fluid volume or serum calcium levels. PTH and FGF-23 are major regulators of PO_4 reabsorption in the proximal tubule (Liu et al. 2007). They are both phosphaturic factors, but as serum FGF-23 levels correlate positively with serum PO_4, the interconnective regulation of phosphate reabsorption by these factors seems to be complicated. Interestingly, insulin increases PO_4 reabsorption by the kidneys, whereas glucocorticoids and glucagon decrease its reabsorption.

2.2.4 Regulation of bone metabolism by growth factors and cytokines

Local and paracrine regulation is of high importance in bone. Bone cells communicate with each other and rapidly respond to changes such as microfractures by expressing or secreting a pattern of growth factors and cytokines which play significant roles in the regulation of bone remodeling.

RANKL. Tumor necrosis factor (TNF) and tumor necrosis factor receptor (TNFR) family proteins are of significance in controlling cell proliferation, cell death, autoimmunity and bone morphogenesis. Major regulators of bone remodeling, RANKL, RANK and OPG are members of this family, being essential for the differentiation and activation of osteoclasts and the initiation of osteoclastic bone resorption (for review, see Väänänen 2005). In addition, RANKL and RANK affect T-cell and dendritic cell functions. RANKL is expressed by mature osteoblasts, mesenchymal cells and hypertrophic chondrocytes. Bone resorption is initiated when RANKL binds to its receptor RANK on the osteoclast plasma membrane. Upon activation by ligand binding, RANK sends signals to the osteoclast cytoplasm via adapter proteins: tumor necrosis factor receptor associated factors (TRAF) 1, 2, 3, 5, and 6 (Darnay et al. 2007). TRAF 6 in particular has been shown to be necessary for the differentiation of osteoclasts by enhancing Src kinase, a molecule essential for osteoclast function (Miyazaki et al. 2004). Activated RANK induces the synthesis of transcription factors NF-κB, AP-1 (c-Fos) and NFATc1, thereby upregulating several genes essential for bone resorption (David et al. 2002). The expression of RANKL by

osteoblasts is increased by various factors such as 1,25 $(OH)_2D_3$, PTH, IL-1, IL-6, IL-11, IL-17, PGE2, TNFα and GCs (Horowitz and Lorenzo 2002; Lacey et al. 1998; Yasuda et al. 1998b). Mice lacking RANKL also lack osteoclasts (Kong et al. 1999b), and injection of soluble RANKL rapidly induces osteoclast formation and bone resorption even in wild-type mice. OPG is a soluble protein produced by osteoblasts that acts as a decoy receptor capable of binding to RANK (Simonet et al. 1997; Tsuda et al. 1997). OPG and RANKL compete for binding to RANK and hence, the RANKL/OPG ratio determines the rate of bone resorption.

M-CSF is a glycoprotein specifically regulating the survival, proliferation and differentiation of monocyte-macrophage lineage cells through the cell surface receptor c-FMS (M-CSFR1). M-CSF, like RANKL, is produced by a variety of bone marrow stromal cells and osteoblasts, but also by T-lymphocytes in response to elevated serum PTH levels and inflammatory molecules such as TNFα and IL-1 (Weir et al. 1996). M-CSF is indispensable for macrophage survival and proliferation as well as for regulating osteoclastogenesis (Tanaka et al. 1993). The significance of M-CSF in osteoclast recruitment is reflected by the op/op mice, which lack functional M-CSF and therefore suffer from severe osteopetrosis due to a lack of osteoclasts (Yoshida et al. 1990). M-CSF strongly suppresses apoptosis of mature osteoclasts by stimulating the extracellular signal-regulating kinase (ERK) and AKT (also known as protein kinase B) pathways. Interestingly, it has been suggested that VEGF could substitute for M-CSF during osteoclastogenesis (Niida et al. 1999).

TNFα is a pro-inflammatory cytokine that has a critical role in the pathogenesis of various inflammatory or immune system mediated diseases, such as rheumatoid arthritis and ankylosing spondylitis. It also seems that TNFα is linked to the development of diabetes mellitus type II (Hotamisligil et al. 1993), and genetic blockade of TNFα action can restore insulin sensitivity *in vitro* and *in vivo* (Uysal et al. 1997). In bone, TNFα secretion is mostly associated with pathological inflammatory conditions (for review, see Teitelbaum 2006), but increased TNFα levels have also been detected in postmenopausal osteoporosis. In an inflamed joint, TNFα is produced and targeted by a variety of cells, and even in physiological conditions, osteoclast precursors and marrow stromal cells express the TNFα receptor p55r (Wei et al. 2005). A part of the effects of TNFα on bone metabolism are thought to be mediated via modulations of the RANKL pathway, resulting in an increased bone resorption rate. In murine osteoclasts, however, it seems that most of the effects of TNFα are mediated via stimulation of IL-1, leading to activation of a complex cytokine and transcription factor pathway (Wei et al. 2005). Whether TNFα alone prompts osteoclast function, is currently controversial, but at least a direct stimulatory effect of TNFα on the differentiation of osteoclast precursors has been observed (Kitaura et al. 2004; Kitaura et al. 2005; Lam et al. 2000). At present a prevailing hypothesis is that the predominant influence of TNFα on osteoclasts occurs via stromal cell population during early stages of an inflammatory disease, but in aggressive disease states, direct effects of TNFα on osteoclasts become more important. Furthermore, there are studies indicating that bone loss observed in many immunological diseases associated with an increased TNFα

expression can be suppressed with TNFα antibodies (for review, see Marotte and Miossec 2008; Zwerina et al. 2005).

The *TGFβ* superfamily includes the BMPs, osteogenic proteins, activins, inhibins and TGFβ isoforms. They are all involved in the coordination of cartilage and bone differentiation during both embryonal development and postnatal life. TGFβ inhibits the proliferation of multipotent hematopoietic progenitors, promotes lineage commitment of neural precursors and suppresses epithelial tumors. TGFβ affects a broad range of cellular activities including control of proliferation and differentiation of several cell types specific to bone, amongst them mesenchymal precursor cells, chondrocytes, osteoblasts and osteoclasts (Joyce et al. 1990; Paley 1990). TGFβ plays a significant role in bone remodeling by suppressing osteoclastogenesis via reduction of RANKL expression by osteoblasts (Quinn et al. 2001). Simultaneously, TGFβ stimulates bone matrix protein synthesis and initiates de novo bone formation (Takeuchi et al. 1993). Interestingly, the bone formation inducing feature of TGFβ might be utilized in plastic surgery in the near future (Ripamonti et al. 2009). A part of the osteogenic activity of TGFβ may be attributed to the production of VEGF. Furthermore, TGFβ has an impact on callus volume and bending strength in experimental fracture models (Lind et al. 1993).

BMPs are important factors regulating embryonal chondrogenesis and skeletogenesis (for review, see Canalis 2009). The BMPs with the greatest osteogenic capacity are BMP-2, -4, -5, -6, -7, and -9. BMP-2 and BMP-7 induce expression of transcription factors RUNX2 and Osterix in MSCs, thereby directing them into osteogenic differentiation. Furthermore, BMP-7-deficient mice show skeletal alterations in the rib cage, hind limbs, and skull (Katagiri et al. 1998). Interestingly, BMP-3 is an exception among the other BMPs while it inhibits BMP-2-induced osteogenic differentiation (Luu et al. 2007). Hence, BMP-3 knockout mice exhibit increased bone density, indicating that BMP-3 antagonizes BMP signaling *in vivo* (Daluiski et al. 2001).

VEGF plays an important role in angiogenesis, endochondral ossification and bone repair after fracture. VEGF is highly expressed by osteoblastic cells *in vitro* (Saadeh et al. 1999; Steinbrech et al. 1999), and studies show that osteoblasts use the Hypoxia-inducible factor α (HIF-α) pathway to sense reduced oxygen tension and transmit signals that affect angiogenic and osteogenic gene programs during bone formation via VEGF (Wang et al. 2007a). VEGF expression by osteoblasts is regulated by a number of cytokines and other factors of the bone microenvironment, including inflammatory cytokines (TGFβ-1, FGF-2, and Platelet-derived growth factor-BB) and hypoxia (Saadeh et al. 1999; Seghezzi et al. 1998; Stavri et al. 1995; Steinbrech et al. 1999). VEGF mRNA and protein are expressed in osteoblasts also during membranous bone repair *in vivo* (Saadeh et al. 1999). Osteoclasts express VEGF receptor 1, which may bind VEGF in order to induce osteoclast recruitment and bone-resorption activity (Niida et al. 2005), and VEGF is indeed thought to act widely on osteoclast differentiation, migration, and activity (for review, see Dai and Rabie 2007). VEGF may also upregulate RANK expression by osteoclast precursor cells (Yao et al. 2006). In cartilage, VEGF is expressed at high levels in hypertrophic chondrocytes and in

mineralized regions of the growth plate cartilage (Carlevaro et al. 2000). This expression has been thought to stimulate chondrocyte proliferation and to promote chondrocyte survival (Dai and Rabie 2007). In addition, VEGF probably has a role in the pathogenesis of osteoarthritis, since it stimulates the formation of neovasculature in inflamed areas (Murata et al. 2007).

Table 1. Top ten cytokines in bone remodeling.

Cytokine	**Main effects on bone remodeling**
RANKL	Induces osteoclast differentiation. Activates bone resorption by osteoclasts. An indispensable cytokine in osteoclasts. (Yasuda et al. 1998; Lacey et al. 1998)
OPG	A decoy receptor that competes for osteoclastic RANK. Prevents osteoclast activation and bone resorption. (Simonet et al. 1997)
M-CSF	Induces osteoclast differentiation. Promotes osteoclast survival. Together with RANKL increases bone resorption. (Tanaka et al. 1993)
TNFα	Promotes osteoclast differentiation. Activates osteoclasts primarily via osteoblasts. Increases bone resorption. Increases osteoblast apoptosis. Linked to inflammation. (Teitelbaum 2006)
TGFβ	Inhibits osteoclast differentiation primarily via osteoblasts. Favors osteoblast differentiation and bone formation. (Quinn et al. 2001)
BMP2, BMP-6, BMP-7	Regulate osteoblast differentiation. Promote bone formation by osteoblasts. Major regulators for embryonal skeletogenesis. (for review, see Canalis 2009)
VEGF	Promotes bone remodeling. Favors osteoclast differentiation and bone resorption. Induces osteoblast differentiation and regulates osteoblast survival. (for review, see Dai and Rabie 2007)
IL-1, IL-6	Stimulate osteoclast fusion, survival and resorption activity both directly and via osteoblasts. May have a biphasic impact on osteoblasts. (Suda et al. 1999)
IGF-1	A major regulator for osteoblast function. Indirectly favors osteoblast differentiation. Promotes bone formation. May enhance osteoclastogenesis and osteoclast function via osteoblasts. (Canalis 2009)
Wnt/β-catenin (the canonical pathway)	Essential for osteoblast function. Deletions of these genes lead to increased osteclastogenesis and absent osteogenesis. Contribute to the pathophysiology of osteoporosis. (Canalis 2009)

2.3 pH regulation in osteoclasts

2.3.1 The significance of extracellular acidosis in calcified tissues

Extracellular pH affects the function of all cells, and it is especially important for osteoclasts. It has been shown that cultured osteoclasts are inactive at extracellular pH levels above 7.3 and show maximum resorption capacity at pH 6.8-6.9 (Arnett and Spowage 1996). Bone resorption is most sensitive to changes in proton concentration at pH 7.1 (Arnett and Spowage 1996). Extracellular bicarbonate content has also been observed to regulate the activity of osteoclasts (Geng et al. 2009). There are controversial studies about the impact of reduced extracellular pH on osteoclast differentiation (Morrison et al. 1998; Shibutani and Heersche 1993) but acidosis has been detected to increase osteoclastic beta-glucuronidase release and overall activity.

Acidosis also decreases osteoblastic collagen synthesis and ALP release (Krieger et al. 1992). Interestingly, isohydric metabolic acidosis and respiratory acidosis have dissimilar effects on osteoblast function. There is a significant decrease in markers for osteoblast activity in metabolic acidosis, whereas no changes can be observed in isohydric respiratory acidosis (Bushinsky 1995b). This indicates that the regulatory role of metabolic acidosis in osteoblasts may not be solely mediated by alterations in proton concentration. Furthermore, acidosis markedly affects growth plate chondrogenesis. A recent study by Das et al. (2010) has shown a clear decrease in *COL1, SOX9* and *VEGF* expression due to acidic conditions in a bioreactor model, while expression of *HIF-1α* is pH independent. Other studies have detected that metabolic acidosis decreases pulsatile growth hormone secretion and reduces serum IGF-1 levels (Kuemmerle et al. 1997), leading to reduced chondrocyte proliferation. In addition, it has been shown that animals with severe metabolic acidosis exhibit reduced expression of chondrocyte IGF-1 mRNA in the epiphyseal growth plate (Green and Maor 2000). Rats exposed to acidic diet exhibit a reduced longitudinal growth rate and thinner growth plates, mainly due to a reduced height of the hypertrophic zone (Carbajo et al. 2001). In addition, acidosis downregulates the synthesis of cartilage matrix proteoglycans and type II collagen and the expression of IGF-1, IGF-1 receptor and PTH receptor at the growth plate. In contrast, an increase in extracellular pH (7.2-7.4) enhances the synthesis of proteoglycans and glycosaminoglycans by articular chondrocytes in a 3D *in vitro* culture, but has no effect on collagen synthesis (Wu et al. 2007).

In order to understand the significance of calcified tissues for the maintenance of systemic pH, it is important to keep in mind the constituents of the inorganic bone matrix. Although the main substance in bone mineral is calcium hydroxyapatite ($[Ca_{10}[PO_4]_6][OH]_2$), which itself has a minor effect on systemic pH, also carbonate ions (CO_3^{2-}) are a common constituent impurity of bone mineral crystals, incorporated in bone during the hydroxyapatite crystal growth (Boskey 2002). In addition, there is a notable bicarbonate reservoir in bone, located in the hydration shell of hydroxyapatite and readily available for systemic circulation. The base reservoir comprised of carbonate and bicarbonate may be utilized to buffer for a descent in the systemic pH, and multiple molecular mechanisms have evolved to ensure the release of alkaline

substances from bone when needed. For instance, in acute metabolic acidosis an immediate release of bone calcium and carbonate through physicochemical dissolution has been detected (Bushinsky et al. 1993), while chronic metabolic acidosis primarily leads to decreased overall bone formation and accelerated bone resorption (Bushinsky 1995a; Krieger et al. 1992). Given these data, it is logical that acidosis has a tremendous impact on overall bone health, and that chronic metabolic acidosis may even account for a major proportion of cases of secondary osteoporosis (Bushinsky 1995a; for review, see Wiederkehr and Krapf 2001).

2.3.2 Key proteins in osteoclastic pH regulation

As mentioned above, osteclasts use acid in order to dissolve minerals from bone matrix. The function of the bone-facing ruffled border in active osteoclasts is to extrude large amounts of ions into the resorption site. Remains of the resorbed bone matrix are endocytosed through this membrane domain. Thus, ruffled border transport proteins play an indispensable role in the bone resorption process, and mutations in these proteins have been shown to lead to deficient bone remodeling (Kornak et al. 2001; Laitala-Leinonen et al. 1999; Laitala-Leinonen and Väänänen 1999; Li et al. 1999). Many of the ruffled border proteins participate in osteoclastic pH regulation, including vacuolar ATPases (V-ATPases) (Laitala-Leinonen et al. 1996; Nakamura et al. 1994, Sundquist et al. 1990), chloride channel ClC7 (Kajiya et al. 2006), potassium-chloride exchanger KCC1 (Kajiya et al. 2006) and the electroneutral sodium/bicarbonate cotransporter NBCn1, recently described by our group and others (Bouyer et al. 2007, II). Furthermore, increasing evidence addresses the role of proteins capable of pH regulation at the non-bone-facing plasma membrane of osteoclasts, such as the anion exchanger (AE) proteins, sodium/proton exchanger 1 (NHE 1) and carbonic anhydrase XIV (CA XIV) (Josephsen et al. 2009, I).

V-ATPases. The vacuolar family of H^+-ATPases are amongst the most important proton transporters in eukaryotes (for review, see Hinton et al. 2007). They were originally identified in intracellular compartments such as endosomes, lysosomes, Golgi-derived vesicles, clathrin-coated vesicles and secretory vesicles, and they have been shown to play an essential role in proton transport across vacuolar or plasma membrane in numerous cell types (for review, see Forgac 2007). Moreover, V-ATPases present within intracellular compartments are important for such cellular processes as receptor-mediated endocytosis, intracellular membrane trafficking, protein processing and degradation. V-ATPases are large, multisubunit protein complexes consisting of a peripheral domain (V1) and an integral domain (V0) that forms the membrane spanning proton channel. Interestingly, the V0 domain of V-ATPases in osteoclasts might play a role in vesicle/vacuole fusion (Peters et al. 2001; Lee et al. 2006), and some V0 subunits bind to actin, allowing interaction between the cytoskeleton and V-ATPases (Holliday et al. 2000; Vitavska et al. 2003).

Osteoclastic V-ATPases migrate from lysosomes to the ruffled border during the first wave of endocytotic vesicle fusion. There they continuously transfer protons across the ruffled border membrane in order to maintain the intralacunar pH at adequately low levels (Blair et al. 1989). V-ATPases are activated by low extracellular pH (Nordström

et al. 1996) while high concentrations of Ca^{2+} and Mg^{2+} as well as a very low extracellular pH inhibit their function *in vitro* (Sakai et al. 2006). In most organisms, the V-ATPase complex is composed of at least 14 separate gene products, with many of the subunits present in multiple isoforms. Several V-ATPase subunit mRNAs are also highly expressed in resorbing osteoclasts (Laitala-Leinonen et al. 1996; Toyomura et al. 2003). Blocking the synthesis of V-ATPases by antisense molecules inhibits bone resorption by isolated rat osteoclasts and disrupts the differentiation of osteoclasts *in vitro* (Laitala-Leinonen et al. 1999; Laitala and Väänänen 1994), and mutations in genes encoding V-ATPase subunits lead to severe osteopetrosis in both mice and humans. Although many V-ATPase subunits are highly expressed in resorbing osteoclasts (Laitala-Leinonen et al. 1996; Manolson et al. 2003), isoform 3 of the 100kDa a-subunit a is typical for osteoclasts (Toyomura et al. 2003; Hu et al. 2005), and mutations in the human a3 subunit lead to autosomal recessive osteopetrosis in more than 50% of affected individuals (Frattini et al. 2000; Kornak et al. 2000; Sobacchi et al. 2001).

ClC7. The CLC gene family of chloride channels includes nine members with important functions in a variety of cells (for review, see Jentsch et al. 1999). Already in 1991, the CLC family member p62 was described in avian osteoclasts (Blair et al. 1991). In 2001, Kornak and others (2001) observed that mice with deficient ClC7 channels have impaired chloride extrusion during osteoclastic bone resorption and, as a result, they suffer from osteopetrosis. ClC7 is expressed already during early mouse embryogenesis in the dorsal root ganglia, the trigeminal ganglion, the eye, and the brain (Brandt and Jentsch 1995; Kornak et al. 2001). Moreover, both embryonal and postnatal osteoclasts express high levels of ClC7 mainly in their late endosomes and lysosomes and at the osteoclast/bone interface (Kornak et al. 2001). ClC7 deficient osteoclasts fail in extracellular acidification and bone resorption but are able to acidify the prelysosomal compartments (Kornak et al. 2001). As a result, mice lacking ClC7 present severe osteopetrosis due to dysfunctional osteoclasts, and this mutation is lethal in mice at the age of 6-7 weeks.

KCC1. K^+/Cl^- co-transporters (KCCs) provide electroneutral movement of K^+ and Cl^- ions driven by their respective chemical gradients. KCCs have been detected in a variety of tissues and cells, including epithelial cells, neurons, the myocardium, skeletal muscle and vascular smooth muscle (Adragna et al. 2000; Greger and Schlatter 1983; Payne et al. 1996; Weil-Maslansky et al. 1994; Yan et al. 1996). Until today, four mammalian KCC isoforms have been cloned (KCC1-4; Gillen et al. 1996; Mount et al. 1999; Su et al. 1999). KCCs have been implicated in cell volume regulation, transepithelial salt transport and regulation of intracellular Cl^- concentration (Gillen et al. 1996). Interestingly, hypotonicity activates KCCs, apparently providing a cellular defence against osmotic shock. A study by Kajiya and others (2006) showed that KCC1 is also expressed in resorbing mouse osteoclasts where it acts as a Cl^- extruder during bone resorption. Human osteoblasts and bovine articular chondrocytes express KCCs in their plasma membrane, likely to be activated together with volume-sensitive Cl^- channels (Hall et al. 1996).

NHE1. Sodium/proton exchangers (NHEs) mediate the electroneutral Na^+ and H^+ transport across plasma membranes. Up to date, nine isoforms (NHE 1 - NHE 9) have been identified within the mammalian NHE family (for review, see Orlowski and Grinstein 2004). They are expressed in all eukaryotic cells and have numerous functions. NHEs regulate intracellular pH (pH_i) and cell volume and initiate changes in the growth or functional state of cells (Grinstein et al. 1989; Hoffmann and Simonsen 1989; Orlowski and Grinstein 1997). Activated by decreased pHi, mammalian NHEs protect cells from intracellular acidification, and hence, cell lines lacking NHE activity are very sensitive to acidosis (Grinstein et al. 1989; Pouyssegur et al. 1984). NHEs also serve as major Na^+ entry pathways in many cell types, regulating both sodium flux and cell volume after osmotic shrinkage (Matsui et al. 2007; Rotin and Grinstein 1989). NHE 1 is required for normal cell growth, proliferation and differentiation (Putney and Barber 2003; Wang et al. 1997). It is important for tumor growth, since tumor cells deficient of Na^+/H^+ exchange either fail to grow or show retarded growth when implanted into athymic mice (Rotin et al. 1989).

NHE activity has been implicated in osteoclast function. Studies by Hall and Chambers (1990) showed that inhibition of NHE decreased bone resorption by rat osteoclasts, and that bone resorption is susceptible to inhibition of NHE during early stages of cell culture (Hall et al. 1992). Later, Kajiya and others (2003) observed that calcitonin inhibits V-ATPase and NHE 1 activity via protein kinase A activation in resorbing rat osteoclasts. In avian osteoclasts, expression of NHE 1 has been detected over the entire basolateral membrane with accumulation at points of attachment to bone (Gupta et al. 1996). However, the exact subcellular localization of NHE 1 in polarized murine osteoclasts is not known. Intriguingly, studies have shown that small and large non-resorbing osteoclasts are more sensitive to amiloride (NHE) inhibition, while large resorbing cells are more sensitive to bafilomycin (V-ATPase) inhibition, and small resorbing osteoclasts are equally inhibited by bafilomycin and amiloride (Lees and Heersche 2000), indicating that proton extrusion mechanisms vary from one osteoclast to another. Recently, another NHE family member, NHE10, was identified in the osteoclast-like cell line RAW 264.7 (Lee et al. 2008).

NHE 1 and NHE 3 proteins are abundantly present in human osteoblast-like UMR-106 cells (Azarani et al. 1995; Mobasheri et al. 1998), whereas NHE 4 is present at the mRNA level. The multiple NHEs in osteoblasts are thought to be needed in the management of ion fluxes that occur during bone formation. NHE has also been shown to play a role in equine chondrocytes, where exposure to low O_2 tension (1%) results in intracellular acidification via inhibition of NHE (Milner et al. 2006). Furthermore, a hypoxia-induced decrease in ROS formation is thought to deactivate NHE in articular cartilage via phosphorylation (Milner et al. 2007).

Ion channels. Osteoclasts possess various ion channels that, for their part, contribute to osteoclast function. Among others, chloride channels (Schlesinger et al. 1997), sodium channels, inward and outward potassium channels (Arkett et al. 1992), calcium-activated potassium channels, nonselective cation channels, and phosphate, proton and calcium channels (van der Eerden et al. 2005; Ypey et al. 1992) have been detected at

various osteoclast plasma membrane domains. It is also of interest to note that the expression of aquaporin 9 increases during mouse osteoclast differentiation from monocytes (Liu et al. 2009), although the significance of this finding remains to be elucidated.

2.3.3 Carbonic anhydrases in osteoclasts

Carbonic anhydrases are ubiquitous zinc-metalloenzymes facilitating the conversion of carbonic acid into carbon dioxide and water, or vice versa. They are highly conserved enzymes present in all life forms. In general, CAs play crucial roles in a variety of cellular functions including carbon dioxide transport, acid-base balance, respiration, bone resorption, ureagenesis, gluconeogenesis and lipogenesis (Parkkila 2000). CAs can be divided into three families of which the alpha-CA family members are expressed in mammals. There are 15 known αCA isoenzymes and a few other CA-like proteins in humans. Four of the active CA isozymes are cytosolic (CA I, II, III, VII and XIII), four are membrane-associated (CA IV, IX, XII and XIV), two are mitochondrial (CA VA and CA VB), and one is secreted (CA VI). Their CA activity and affinity to sulfonamide inhibition are described in Table 2. Furthermore, CA-related proteins (CA-RP-VIII, CA-RP-X and CA-RP-XI) and receptor-type protein-tyrosine phosphatases (RPTP β and γ) have been characterized in humans, but their enzymatic activity is deficient due to a deformed active site in the enzyme. In addition to promoting CO_2 hydration, CAs may be capable of facilitating other reactions, such as the hydration of cyanate to carbamic acid, or hydration of cyanamide to urea, aldehyde hydration to geminal diols and hydrolysis of carboxylic or sulfonic esters. Two main classes of CA inhibitors exist: metal complexing anions such as thiocyanate, and unsubstituted sulfonamides such as acetazolamide (for review, see Pastorekova et al. 2004).

CA I is a ubiquitous isoenzyme expressed in red blood cells, the intestinal epithelium, vascular endothelium, corneal epithelium and the lens of the eye (Carter and Parsons 1971; Spicer et al. 1979). Interestingly, *CA I* has two promoters, one erythroid and another non-erythroid (Brady et al. 1991). As for CA V, there are in fact two genetically different isoforms, CA VA and VB, which are differentially expressed in humans (Fujikawa-Adachi et al. 1999b). CA VA can be found predominantly in the liver, whereas CA VB is expressed in skeletal and heart muscles as well as the gastrointestinal tract. CA VI is a secretory isoenzyme expressed in the salivary and mammary glands in humans (Fujikawa-Adachi et al. 1999c). Its function in the salivary glands is thought to be related to taste sensing and protection from caries. CA VII is expressed in the central nervous system both in mice and in humans, where it possibly participates in producing cerebrospinal fluid (Halmi et al. 2006). CA XIII is expressed in human at least in the thymus, small intestine, spleen, prostate, ovary, colon, and testis (Lehtonen et al. 2004), but its role is not yet well known. CA XV is the most recently characterized isoenzyme that is enzymatically functional in mammals except in primates, where it is totally absent and likely to be substituted by other CAs (Hilvo et al. 2008).

Table 2: Description of some properties of human CA isoenzymes.

Isoenzyme	Enzymatic activity	Affinity for sulfonamides
CA I	low/moderate	moderate
CA II	high	very high
CA III	very low	very low
CA IV	high	high
CA V	moderate/high	high
CA VI	moderate	low/moderate
CA VII	high	high
CA IX	high	high
CA XII	low	low
CA XIII	low/moderate	high
CA XIV	high	high

CA II is the most important isoenzyme present in the osteoclast cytoplasm, providing a continuous supply of protons for the acidification of the resorption lacuna. CA II expression is most abundant in the cytoplasm and on the inner surface of the ruffled border of osteoclasts (Anderson et al. 1982; Zheng et al. 1993), and it is an early character of osteoclast differentiation (Karhukorpi 1991; Laitala and Väänänen 1993; Väänänen and Parvinen 1983). CA II activity is essential for bone resorption, and loss of CA II results in severe osteopetrosis in both humans and mice (Hall and Kenny 1987; Hall et al. 1991; Hott and Marie 1989; Kenny 1985; Margolis et al. 2008). A decrease in the extracellular pH of osteoclasts increases intracellular CA II expression (Schwartz et al. 1994). Furthermore, CA II seems to act as a mediator for hormones increasing bone resorption and osteoclast formation, since both PTH and $1,25(OH)_2D_3$ stimulate CA II expression (Hall and Kenny 1985; McSheehy and Chambers 1986; Silverton et al. 1987). In fact, inhibition of CA II abolishes the effect of PTH on bone (Hall and Kenny 1987).

There is plenty of evidence that CA II has a key role in regulating pH_i in osteoclasts and in promoting resorption activity at different pH_e levels. First of all, blocking CA II expression with antisense RNA and DNA inhibits bone resorption (Laitala and Väänänen 1994; 1993). CA II expression is stronger in actively resorbing than in non-resorbing osteoclasts (Asotra et al. 1994), and the unspecific CA inhibitor acetazolamide blocks bone resorption (Hall and Kenny 1987; Kenny 1985; Lehenkari et al. 1998). The importance of CA II to osteoclasts may also be highlighted with the fact that the first genetic mutation discovered to be responsible for osteopetrosis was a *CA II* deficiency which also causes renal tubular acidosis and cerebral calcification in humans (Ohlsson et al. 1986; Ohlsson et al. 1980; Sly et al. 1983, Sly and Hu 1995). Interestingly, the role of CA II in the regulation of pH_i seems to vary at different stages of osteoclast activity (Lehenkari et al. 1998).

The action of intracellular CA II is often completed by other CA isoenzymes, ion channels and ion exchangers (for review, see Sterling et al. 2001). For instance, Vince and Reithmeier (1998) found a direct, specific interaction between CA II and the erythrocyte Cl^-/HCO_3^- exchanger (AE protein) and noted that CA II is ideally positioned to catalyze CO_2 hydration and to supply the Cl^-/HCO_3^- exchanger with bicarbonate. Moreover, CA II has been detected to increase the activity of the sodium/bicarbonate transporter NBCe1 and even to physically connect to kNBC1 to facilitate its function (Loiselle et al. 2004; Pushkin et al. 2004; for review, see Sterling and Casey 2002). This kind of molecular interaction between CA II and other proteins, also known as a bicarbonate transport metabolon, functions to enhance the transport of bicarbonate through biological membranes. Membrane-bound CAs have also been suggested by our group and others to be integral parts of these metabolons (Casey et al. 2009; Morgan et al. 2007; Sterling et al. 2002; for review, see McMurtrie et al. 2004, I). During the last few years it has become evident that bicarbonate metabolons are necessary for optimizing bicarbonate transport and that they are a universal molecular mechanism present in many types of cells and organisms.

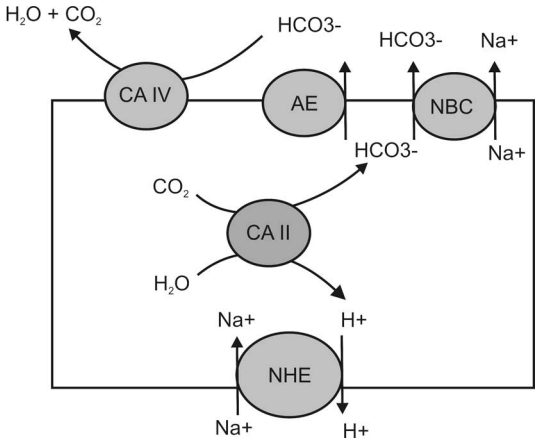


Figure 3: A model for a cellular bicarbonate transport metabolon (for review, see Sterling and Casey 2002). CA IV = carbonic anhydrase IV, AE = a bicarbonate transport protein (AE protein), NBC = a bicarbonate/sodium cotransporter, CA II = carbonic anhydrase II, NHE = a sodium/hydrogen cotransporter.

CA III is an isoenzyme mainly found in the skeletal muscle. It comprises about 8% of the soluble protein in red skeletal muscle, and in male rat liver and adipocytes it constitutes up to 25% of the total cytosolic protein (Carter 1991). It is also present in some other tissues and cell types although in a much smaller quantity (Spicer et al. 1990). CA III is traditionally regarded as a cytosolic enzyme, but there have been histochemical observations of its nuclear localization (Dodgson et al. 1993; Räisänen et al. 1999). The hydratase activity of CA III is very low, but it has been suggested to have carboxyl esterase and phosphatase activities, indicating that it might act as a tyrosine phosphatase (Cabiscol and Levine 1996; Koester et al. 1981). We have studied the role of CA III in embryonal mouse chondrogenesis (III).

Membrane-bound CAs. In addition to intracellular CA isoenzymes, there are four active membrane-bound isoenzymes. The active CA domain of CA IV, CA IX, CA XII and CA XIV is extracellular. Of these, CA IV is linked to the cell membrane with a glycosyl-phosphatidylinositol anchor, whereas the other membrane-bound isoforms are integral membrane proteins. CA IV is expressed in various cell types, such as in the

kidney proximal tubules, astrocytes and rat muscle cells (Purkerson et al. 2007; Sender et al. 1994; Svichar et al. 2006). CA IV is considered to actively participate in pH regulation, for instance in the regulation of urine acidification (Schwartz et al. 2000). Additionally, studies have linked CA IV to bicarbonate transport metabolons, and CA IV and AE1 have even been shown to interact directly (Sterling et al. 2002). CA IX is rarely found in healthy mammals except for some parts of the gastrointestinal tract (Kivelä et al. 2005), while it is widely present in various cancer cell types, such as carcinomas of the cervix uteri, esophagus, kidney, lung and breast. In fact, CA IX expression is specifically associated with aggressive cancer types. It is thought to support tumor survival possibly by acidifying tumor surroundings (Lee WY et al. 2008). The expression of CA IX in tumors is markedly stimulated by hypoxia, which has led to its use as a tumor marker also in the clinical practice (Takacova et al. 2007). CA IX is widely considered as a potential drug target since its inhibition might prevent tumor growth (Winum et al. 2009). In contrast, CA XII is an active, basolateral isoenzyme, the catalytic properties of which are similar to those of the high-activity CA IV (Ulmasov et al. 2000). The expression of CA XII has been demonstrated widely: in the adult human kidney, colon, prostate, pancreas, ovary, testis, lung and brain (Kallio et al. 2006). However, CA XII was initially discovered in renal cell cancers and a lung cancer cell line, and therefore it is not surprising that also CA XII is associated with certain cancers. In fact, on the mRNA level, CA XII is regulated by the VHL tumor suppressor gene (Ulmasov et al. 2000).

CA XIV is one of the more recently found membrane-bound CAs which, intriguingly, can be located either at the apical or basolateral membrane of cells. It is an integral membrane protein with an extracellular NH_2-terminal catalytic domain, a membrane-spanning segment of 22 amino acids, and a short intracellular COOH-terminal segment with several potential phosphorylation sites (Fujikawa-Adachi et al. 1999a; Mori et al. 1999). CA XIV is expressed in the kidneys at the most important bicarbonate reabsorption site, i.e. the proximal convoluted tubule (Kaunisto et al. 2002; Mori et al. 1999), and it has been suggested to function in bicarbonate reabsorption by type A intercalated cells. Moreover, CA XIV activity has been detected by Northern blotting in several human, mouse and rat tissues (Fujikawa-Adachi et al. 1999a; Mori et al. 1999), including the heart, lung, liver, skeletal muscle, and neuronal cell membranes and axons of human and mouse brain, as well as the pigment epithelium of the retina. The functions of CA XIV are poorly known but at least in the murine brain it seems to play a role in buffering the extracellular space (Shah et al. 2005) and augmenting the operation of AE3 in hippocampal neurons (Svichar et al. 2009). In addition, CA XIV participates in producing the normal retinal light response (Ogilvie et al. 2007). *CA XIV* knockout mice have normal viability and fertility (Shah et al. 2005). We have studied the expression and role of membrane-bound carbonic anhydrases, especially CA IV and CA XIV in rat osteoclasts (I).

2.3.4 Bicarbonate transporter proteins in osteoclasts

Bicarbonate transporter families include the AE family of sodium-independent Cl^-/HCO_3^- exchangers, the NBC family of Na^+/HCO_3^- cotransporters and the sodium-dependent Cl^-/HCO_3^- exchanger family. All of these families possess related amino

acid sequences and therefore form a superfamily called Solute carrier family 4 (*SLC4A*, for review see Romero et al. 2004; Soleimani and Burnham 2001; Sterling and Casey 2002).

AE proteins. The AE bicarbonate/chloride transporter family is encoded by three genes, *AE1, AE2, and AE3*, all of which give rise to multiple mRNA transcripts. AE1 (also known as band 3) is expressed in multiple tissues, such as erythrocytes, the kidney and testis (Brosius et al. 1989; Kudrycki and Shull 1989). AE2 was originally isolated from the human erythroleukemic cell line K562 and subsequently from several other tissues (Demuth et al. 1986). In turn, AE3 is predominantly found in excitable tissues including the brain (Kopito et al. 1989), heart (Linn et al. 1992) and retina (Kobayashi et al. 1994). Interestingly, in addition to bicarbonate, AE proteins are able to transport a range of other small inorganic and organic anions (Jennings 1989) as well as small organic phosphates. AE1 transports anions over a broad pH range, whereas AE2 and AE3 are pH-sensitive, and their function is inhibited in acidic conditions (Sterling and Casey 2002).

Proton production by CA II and secretion by V-ATPases in osteoclasts generates a load of cytoplasmic base, mainly HCO_3^-. Moreover, bicarbonate and carbonate are dissolved in remarkable amounts from the bone matrix during resorption. This bicarbonate must be transported away from the resorption site and out of osteoclasts in order to maintain cytoplasmic pH at the physiological range. Bicarbonate/chloride exchangers (AE proteins) are utilized in this process (Bastani et al. 1996; Hall and Chambers 1989; Teti et al. 1989). Avian osteoclasts express the AE1 protein, whereas mouse osteoclasts apparently express both AE2 and AE3 (Josephsen et al. 2009; Kajiya et al. 2006; Teti et al. 1989). The function of AE proteins in osteoclasts is ATP- and sodium-independent and may be blocked by anion-exchange inhibitors 4,4'-diisothiocyano-2,2'-stillbene-disulfonic acid (DIDS) and a chloride-specific channel blocker diphenhydramine hydrochloride (DIPH) (Hall and Chambers 1989). There are also studies showing that the recovery of osteoclasts from an alkaline load is slowed down in a chloride-free media (Teti et al. 1989). Even more importantly, *AE2* knockout mice suffer from severe osteopetrosis, and their osteoclasts are enlarged and unable to form the ruffled border (Josephsen et al. 2009). Such findings emphasize that Cl^-/HCO_3^- exchange is essential for the acidification of the resorption compartment.

As discussed above, AE proteins are thought to be important components of bicarbonate transport metabolons, aiming at an efficient flux of bicarbonate over biological membranes (for review, see Sterling et al. 2001). Several lines of evidence support the participation of AEs in these metabolons: for instance, CA II can be co-immunoprecipitated with solubilized AE1, and lectin-mediated agglutination of AE1 in erythrocytes leads to a similar redistribution of CA II (Vince and Reithmeier 1998). Furthermore, CA II has been shown to be able to bind the C terminus of AE1 (Vince and Reithmeier 1998).

NBCn1. A Na/HCO_3^- cotransporter (NBC) was first described in the kidney proximal tubule (Boron and Boulpaep, 1983). Several isoforms of NBC have been distinguished

since, and four isoforms are expressed in humans. In addition, several 5´- or 3´-terminal splice variants have been cloned for most NBC isoforms, although it is not currently known whether each variant has a unique localization and function. The nomenclature within the NBC family is quite confusing for historical reasons, and hence the different names and splice variants used of the family members are briefly introduced in Table 3. All of the NBC family members are integral membrane proteins and possess the capacity for transporting bicarbonate. The NBC family members are distinguishable as regards electrogenity and their capacity for carrying anions or cations besides bicarbonate. Regarding NBCE, it is currently not clear whether it is a Na^+-dependent chloride/bicarbonate exchanger or an electroneutral Na^+/HCO_3^- transporter. NBCs are critical in multiple functions such as the regulation of cellular volume, HCO_3^- reabsorption by the kidney, HCO_3^- secretion by the pancreas and the regulation of intracellular pH.

Table 3: Nomenclature in the NBC bicarbonate transporter family.

Isoform (gene)	Alternative names/splice variants	Membrane localization	Predominant substrate
NBC1 (*SLC4A4*)	NBCe1, kNBC1, pNBC1, hNBC1, hhNBC1	basolateral	sodium, bicarbonate/carbonate
NBC2 (*SLC4A7*)	SBC2, NBCn1, NBC3, mNBC3, retina NBC2, NBC2b	apical or basolateral	sodium, bicarbonate
NBC3 (*SLC4A8*)	NDCBE, kNBC3, NDAE1	likely basolateral	sodium, bicarbonate, chloride
NBC4 (*SLC4A5*)	NBCe2, NBC4a, NBC4b	unknown	sodium, bicarbonate/carbonate
NCBE (*SLC4A10*)	NBCn2	likely basolateral	sodium, bicarbonate, chloride

The electroneutral sodium/bicarbonate transporter NBCn1 was originally cloned from rat smooth muscle cells (Choi et al. 2000) and it has been shown to be ~92% identical to human NBC2 and ~50–55% identical to NBC1, respectively. NBCn1 has a wide expression profile with promoter activity in tissues including kidney thick ascending limb and medullary collecting duct epithelial cells, vascular smooth muscle cells and endothelial cells, the epithelial lining of the kidney pelvis, choroid plexus epithelial cells, hippocampus, duodenal enterocytes, and the retina (Boedtkjer et al. 2007). In earlier studies, it was demonstrated that the NBCn1 protein is present in the basolateral domains of thick ascending limb cells of the outer medulla of rat kidney (Vorum et al. 2000). Moreover, studies have shown that chronic metabolic acidosis is associated with a marked increase in the abundance of NBCn1 in the medullary thick ascending limb cells, indicating that NBCn1 could play a role in ammonium (NH^{4+}) reabsorption, medullary accumulation, and urinary excretion, as well as basolateral bicarbonate transport into thick ascending limb cells (Kwon et al. 2002). Recent data have also

shown that NBCn1 expression in the brain is induced by chronic metabolic acidosis and that N-methyl-D-aspartic acid inhibits this induction, associating NBCn1 expression with glutamate in the brain (Park et al. 2010). Interestingly, Chen and colleagues (2007) have previously shown that chronic exposure to hypoxia in rats decreased brain NBCn1 levels significantly, and they hypothesized that this reduction may be important for minimizing energy consumption by the cells.

In bone, CSF-1 promotes osteoclast survival via an NBCn1-dependent mechanism in non-resorbing rat osteoclast-like cells (Bouyer et al. 2007). CSF-1 caused a substantial increase in pH_i via the activation of NBCn1, which in turn reduced caspase activity in these cells. We have studied NBCn1 in human and rat osteoclasts and showed that NBCn1 is not only located at the osteoclast ruffled border but also plays a significant role in bone resorption (II).

2.3.5 Metabolic bone disease in chronic acidosis and renal failure

Metabolic acidosis is a condition where the systemic pH drops under the normal range of 7.35-7.45 in circulation because of accumulation of acid, leading to numerous consequences as the body attempts to restore homeostasis. It is a common phenomenon in severe diseases such as (acute/chronic) renal failure, sepsis, diabetes mellitus, ischemia, anemia and chronic diarrhea, which all involve either overproduction of organic acids such as lactic and ketoacids or decreased elimination of acid. Metabolic acidosis leads to alterations in the hormonal balance which include a decrease in IGF-1 levels due to peripheral growth hormone insensitivity, primary hypothyroidism and hyperglucocorticoidism (Jandziszak et al. 2000). Moreover, metabolic acidosis induces a negative calcium balance with hypercalciuria and a tendency to develop kidney stones. Natriuresis occurring due to inhibition of tubular sodium reabsorption induces extracellular volume contraction and results in secondary hyperaldosteronism (Henger et al. 2000). Hypophosphatemia due to renal phosphate wasting may also develop. Metabolic acidosis in both humans and rats results in growth retardation and increases protein breakdown in skeletal muscle (Kalhoff et al. 1993; May et al. 1986). The consequences of metabolic acidosis in calcified tissues are complicated and related to other regulators of bone and mineral metabolism. In general, metabolic acidosis induces hypercalciuria due to release of calcium from bone and decreased renal tubular calcium reabsorption, renal phosphate depletion and hypophosphatemia, an increase in serum $1,25\text{-}(OH)_2D_3$ and a corresponding decrease in PTH, and hypocitraturia. Negative calcium balance and phosphate depletion combine to induce a metabolic bone disease that exhibits features of both osteoporosis and osteomalacia and, on the whole, bone resorption is increased and bone formation is decreased. New ways of treating the clinical bone manifestations of metabolic acidosis are warranted, and some promising trials are ongoing.

Chronic kidney disease means a progressive loss of renal function over a period of months or years. This syndrome leads to multiple changes in one´s health, including problems with the circulation and fluid homeostasis, and accumulation of harmful substances such as urea. Chronic renal failure also causes alterations in bone metabolism with features depending on the stage of the disease. In the early stages of

kidney disease, the levels of potent phosphaturic hormones such as FGF-23 and PTH in serum increase (Quarles 2003). A rise in serum FGF-23 precedes the increase in PTH secretion and aims at maintaining serum phosphate within the normal range by boosting renal phosphate excretion. As the glomerular filtration rate falls beneath 20–30 ml/min, FGF-23 secretion fails to maintain normal serum phosphate concentrations (Ritz and Gross 2005). The resulting hyperphosphatemia, $1,25(OH)_2D_3$ deficiency and hypocalcemia stimulate PTH synthesis and secretion. PTH, in turn, acts to normalize plasma calcium and phosphate concentrations, which is achieved by increasing bone turnover and phosphate excretion. This cascade leads to an atypical metabolic bone disease with either normal, high or low bone mineral density, showing features of both osteoporosis and osteomalacia. Currently, the treatment of the metabolic bone disease includes medication to normalize the perturbations in PTH-calcium/phosphate-vitamin D axis and bone turnover rate. Drugs such as aluminium- and calcium-containing phosphate binders and calcitriol suppress PTH secretion by acting directly on the parathyroid gland, or indirectly by reducing serum phosphate and increasing serum calcium levels (Diaz-Corte et al. 2001). However, agents normally used to treat osteoporosis, such as bisphosphonates, may not be beneficial in chronic kidney disease but, in fact, may lead to severe osteomalacia through reduced bone turnover. Thus, the treatment of bone disease due to renal failure is usually focused on achieving normal bone turnover that may be accomplished by maintaining adequate levels of anabolic PTH and vitamin D receptor agonists.

2.4 Oxidative metabolism in calcified tissues

2.4.1 Significance of hypoxia in cellular metabolism

Oxygen is required by most cells for the production of adequate amounts of ATP for metabolic activities. Deprivation of oxygen, i.e. hypoxia, is considered a harmful condition that occurs in humans due to a variety of disease conditions including diseases of the lungs and circulatory system and a number of other systemic diseases (for review, see Semenza 2000). Nevertheless, hypoxia may be an important and beneficial local phenomenon for instance during embryonal development. Among others, oxygen deprivation is related to the neural tube closure, mediation of apoptosis and purposeful morphological development during gestation (Chen et al. 1999; Genbacev et al. 1997; Iyer et al. 1998). In a cellular system, hypoxia may present either harmful or beneficial consequences but, in any case, it has a remarkable influence on cellular metabolism. Oxygen sensing by cells is mediated via various mechanisms. Current data indicates that direct and rapid inhibition of the function of ion channels (such as potassium and calcium channels) is one mechanism that mediates the downstream effects of lowered oxygen (for review, see López-Barneo et al. 2009). Hemo-oxygenase-2 (Williams et al. 2004), NAPDH oxidase, AMP kinase and mitochondria (Wyatt and Buckler 2004) have also been suggested to have possible roles in oxygen sensing.

Most effects of hypoxia on cells are thought to be mediated via its influence on gene expression through the hypoxia-inducible factor (HIF) pathway. Activation of the HIF pathway is prompted by hypoxic conditions, which result in an interaction with enzymes and transcription factors regulating anaerobic metabolism, immunological

responses, vascularization, pH regulation and tissue growth (Dunwoodie 2009). HIF-1α is an extremely important mediator of cellular metabolism, and it is constitutively expressed by all cells and crucial to cellular homeostasis. In fact, HIF-1α knockout mice are not viable (Ryan et al. 1998). One of the best known interactions of HIF-1α is its role in the activation of *VEGF* transcription in hypoxic cells (Carmeliet et al. 1998). HIF-1α also activates numerous other genes such as NF-κβ, CA IX, CA XII and erythropoietin (Holotnakova et al. 2007). Moreover, proliferation of tumor cells is facilitated by HIF-1α activation: the microenvironment surrounding tumors is extremely hypoxic, and activation of the HIF-1α pathway leads to increased angiogenesis and an increased oxygen supply to the area (Carmeliet et al. 1998; Ryan et al. 1998). HIF-2α activates partly overlapping and partly distinct sets of target genes with HIF-1α (Hu et al. 2003), and in general both HIF-1α and HIF-2α play roles in the regulation of angiogenesis, whereas glycolytic genes are predominantly regulated by HIF-1α. HIF-3α may, by contrast, provide a negative feedback loop for the HIF-regulated gene activation pathway, since HIF-3α has been shown to compete for HIF-1β and to dimerize with it (Makino et al. 2001).

Activated HIF is a dimeric protein complex. In hypoxic conditions, HIF-1α and/or HIF-2α translocate to the cell nucleus and dimerize with the constitutively expressed HIF-1β (also known as Aryl hydrocarbon receptor nuclear translocator, ARNT), and this complex binds to DNA at sites represented by the consensus sequence 5'-RCGTG-3', i.e. at hypoxia-responsive elements (Semenza et al. 1996). The HIF-1α subunit is unique to HIF-1, whereas HIF-1β can also dimerize with HIF-2α and other basic helix-loop-helix proteins. Activity of the HIF transcription factor can be regulated via enzymatic control of the abundance and activity of HIF-α subunits by various genes. However, the most important regulator of the HIF complex is the von Hippel-Lindau protein (pVHL). Under normoxia, HIF-α binds to pVHL, which is part of an E3-ubiquitin ligase complex that targets HIF-α for proteasomal degradation (Jaakkola et al. 2001). Hence, in normoxia HIF-1α has a short half-life of less than 5 minutes (Yu et al. 1998). By contrast, in hypoxia prolyl-hydroxylation is inhibited and HIF-α is stabilized, allowing intracellular accumulation and interaction with HIF-1β. Loss of pVHL function also results in HIF-α stabilization that is independent of oxygen levels (Haase 2006). Furthermore, HIF-α subunits can be regulated via transcriptional inactivation following asparaginyl hydroxylation by factor inhibiting HIF (FIH) (Mahon et al. 2001). An interaction network of transcription factors associated with HIF-α is described in Figure 4.

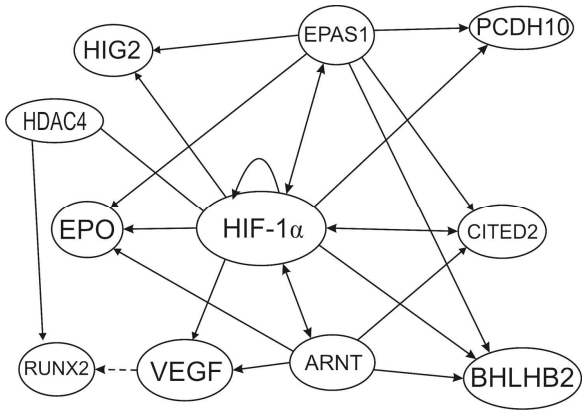


Figure 4: An interaction network of transcription factors linked to HIF-1α. Arrows show the direction of the interactions. Abbreviations: HIG2, hypoxia-inducible protein 2 (Wang et al. 2005); EPAS1, endothelial PAS domain protein 1 (Maemura et al. 1999); CITED2, Cbp/p300-interacting transactivator (Tien et al. 2004); BHLHB2, basic helix-loop-helix domain containing, class B, 2 (Wang et al. 2005); ARNT, hypoxia-inducible factor 1β (Suzuki et al. 2001); VEGF, vascular endothelial growth factor (Sanchez-Elsner et al. 2001); EPO, erythropoietin (Lee et al. 2004); HDAC4, histone deacetylase 4 (Qian et al. 2006); RUNX2, runt-related transcription factor 2 (Namba et al. 2000); PCDH10, protocadherin 10 precursor. Source: Ingenuity Pathway Analysis knowledge base (www.ingenuity.com).

2.4.2 Reactive oxygen species and antioxidant systems in cellular metabolism

Reactive oxygen species are O_2-derived free radicals, including hydrogen peroxide (H_2O_2), superoxide anion ($O2^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($RO_2^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$). They predominantly arise in mitochondria as byproducts of oxidative phosphorylation, a process that is responsible for ATP production in the cells in aerobic conditions. At physiological levels, ROS act as redox messengers that mediate intracellular signaling, whereas in oxidative conditions, excessive ROS induce oxidation of cellular macromolecules such as lipids and nucleic acids, inhibit protein function and cause direct damage to mitochondrial DNA. In other words, excess ROS production promotes cellular apoptosis by activating either direct or indirect mitochondrial apoptotic pathways. It has become evident that ROS are key molecules in aging and in the pathophysiology of various diseases such as atherosclerosis, diabetes mellitus and neurodegenerative diseases (Mani et al. 2007).

Several systems have evolved to resist the destructive impact of excess ROS on cellular metabolism, and these systems are mainly located in intracellular redox compartments. The cellular redox systems include superoxide dismutase and catalases, thiol-reducing buffers such as glutathione (GSH) and thioredoxins, pyridine nucleotides such as NADPH, and some other molecules, such as vitamin C and E and the recently found 3β-hydroxysteroid-Δ24 reductase DHCR24 (Lu et al. 2008; for review see Kalinina et al. 2008). One of the most important molecules within this group is glutathione which is the most abundant free thiol in eukaryotic cells. In physiological conditions, GSH is present mostly in a reduced form, whereas in the presence of excess ROS, GSH is oxidized into glutathione disulfide (GSSG), and this reaction may be facilitated by GSH peroxidase (for review, see Dickinson and Forman 2002). As GSSG is formed, an electron is donated to the ROS, reducing its activity and preventing its harmful oxidizing action on proteins or DNA. If the cell is exposed to a reasonable amount of oxidative stress, oxidation of GSH to GSSG is reversible and

GSSG may be converted back to GSH. However, there are data showing that if a certain treshold of oxidative stress is exceeded, GSSG formation within minutes of this stress may trigger mitochondrial proapoptotic signaling, and apoptosis may occur hours later even if the amount of reduced GSH returns soon to normal levels (Pias and Aw 2002). GSSG can be reduced back to GSH in a reaction facilitated by GSH reductase, where NADPH acts as a cosubstrate (for review, see Kalinina et al. 2008). NADPH and also thioredoxins play important roles in the maintenance of homeostatic redox state in cells, since they are able to provide the reducing equivalents needed for several biosynthetic reactions such as cholesterol synthesis and cytochrome 450 hydroxylation. Moreover, conversion of $NADP^+$ to NADPH is a necessary reduction reaction in the pentose phosphate pathway, an alternative energy source to glycolysis. Mitochondrial oxidative phosphorylation, including the reduction reaction of NAD^+ to NADH is downregulated during oxidative stress, and the released mitochondrial reducing equivalents are used in favor of $NADP^+$ reduction, converting the primary cellular source of energy to glycolysis (Singh et al. 2007).

2.4.3 Oxidative metabolism, oxygen sensing and hypoxia in bone

Osteoclasts are highly active cells regards cellular metabolism. They contain abundant mitochondria, the function of which is to provide cells with ATP. For instance, maintaining ion flux during acidification of the resorption lacuna demands an efficient supply of energy. Therefore, metabolic enzymes involved in the citric acid cycle and oxidative phosphorylation are upregulated during osteoclast differentiation (Czupalla et al. 2005). Moreover, exogenous ATP has been observed to stimulate osteoclast formation and resorption (Morrison et al. 1998). Kim and colleagues (2007) observed that glucose metabolism, including glycolysis and mitochondrial respiration, was accelerated at an early stage of RANKL-induced osteoclast differentiation. They also found that primary metabolic routes in osteoclasts can be altered according to their activation status. The significance of mitochondrial function in osteoclasts was also addressed by an interesting study by Oursler et al. (2005) suggesting that the mitochondrial apoptotic pathway observed in many other cell types is also active in osteoclasts. This study showed that if M-CSF and RANKL levels decrease in the bone microenvironment, an increase in H_2O_2 formation by osteoclasts resulted in an increased ROS production rate which, in turn, led to activation of the apoptotic pathway. Recently, a report was published on the effects of hyperoxia on osteoclasts (Yamasaki et al. 2009). According to this report, hyperoxia may prolong osteoclast precursor cell formation from $CD14^+$ cells as a result of increased M-CSF production by supporting cells. Thus, it can be concluded that oxidative metabolism is of importance in active osteoclasts.

During osteoblastic differentiation, shifts in metabolic pathways have been observed (Komarova et al. 2000). When osteoblasts are differentiated from cells derived from calvaria, cellular metabolic activity, glycolysis and respiration fluctuate during sequential differentiation stages. In the undifferentiated period, the major energy source depends on glycolysis. Nevertheless, the rate of cellular respiration is markedly increased during the early stages of differentiation to allow active proliferation. Both glycolysis and respiration are then enhanced during middle and late stages of osteoblast

maturation. Possibly linked to this metabolic switch during osteoblastic differentiation, a work by de Souza Malaspina and colleagues (2008) detected a link between TRAcP expression and the levels of reduced glutathione, an important indicator of cellular oxidative state, in osteoblast-like cells (de Souza Malaspina et al. 2008). Oxygen sensing in bone is predominantly thought to be mediated by osteoblast-lineage cells, although the exact mechanisms are not well known at present. Some suggestions have been made about the possible role of heme-containing molecules in monitoring immediate changes in oxygen levels, and the signal is thought to be transmitted further by the HIF-VEGF pathway (Steinbrech et al. 2000; Steinbrech et al. 1999). Current knowledge indicates that this osteoblast-dependent oxygen signaling route may also be responsible for the initiation of bone fracture repair (Glowacki 1998).

Hypoxia is likely an ordinary *status quo* in bone tissue, since oxygen tension (pO_2) inside a normal mandible has been shown to reach 8.6% at most (Maurer et al. 2006), and some measurements have indicated even lower oxygen levels in the bone marrow. It has been proposed that hypoxia may also represent a mechanotransduction pathway, since numbers of hypoxic or HIF-1-expressing osteocytes are increased *in situ* in response to mechanical unloading (Dodd et al. 1999; Gross et al. 2001). In the same time, another study indicates that osteocytes deprived of mechanical loading increase osteopontin (OPN) expression, which is thought to activate disuse-induced bone resorption by osteoclasts (Gross et al. 2005). Thus, it is possible that hypoxia and the HIF pathway possess key roles in the development of disuse-induced osteoporosis, but this issue has not been thoroughly studied. Evidence for the effects of hypoxia on cultured osteoblasts seem to be controversial. Some studies suggest increased proliferation of osteoblasts with decreased ALP activity and collagen synthesis due to hypoxia (Lennon et al. 2001, Matsuda et al. 1998; Tuncay et al. 1994), while others show decreased proliferation with increased syntheses of ALP, IGF-2 and TGFβ1 (Steinbrech et al. 1999). Nevertheless, it is widely accepted that the stimulation of VEGF synthesis in hypoxia via the HIF-pathway represents a basic homeostatic response to low oxygen levels by osteoblasts (Akeno et al. 2001). Osteoblasts express both HIF-1α and HIF-2α and, interestingly, VHL-deficient mice (overexpressing HIF-1α and HIF-2α) have a significantly increased bone volume and skeletal vascularity when compared to controls (Wang et al. 2007 a,b). Furthermore, the development of long bone vascularization is disturbed in a similar manner in mice with conditional knockouts of HIF-1α or HIF-2α, associating both of these factors to normal skeletal angiogenesis (Shomento et al. 2010). On the other hand, in the same study HIF-1α deficient mice demonstrated markedly decreased trabecular bone volume, reduced bone formation rate, altered cortical bone architecture and impaired osteoblast proliferation but by contrast, HIF-2α deficient mice present only a modest decent in trabecular bone volume and normal osteoblast proliferation (Shomento et al. 2010). Hence, it seems clear that both HIF-1α and HIF-2α are necessary for normal long bone development and in osteoblasts, but they partly exert distinct functions.

Hypoxia acts in general as a stimulator for the formation and activation of cells of the monocyte-macrophage lineage (Broxmeyer et al. 1990). Hence, it is no surprise that significant stimulation of osteoclast formation and bone resorption activity may be

observed in hypoxic cultures exposed to 0.2% oxygen (Arnett et al. 2003). In mouse calvaria cultures, exposure to hypoxia resulted in a 5-fold stimulation of osteoclast-mediated calcium release through increased resorption, an effect equivalent to that of PGE2 (Arnett et al. 2003). Further evidence shows that exposure of RAW 264.7 cells to hypoxia, leading to increased ROS production and disruption of mitochondrial transmembrane potential, induces stress signaling pathways that involve the activation of calcineurin and Ca^{2+}-responsive factors and, thus, activates osteoclastogenesis (Srinivasan and Avadhani 2007). It has been proposed that some of the osteoclast activation caused by hypoxia is mediated via the secretion of IGF-2 by non-osteoclastic cells (Fukuoka et al. 2005). A recent study confirmed the role of HIF-1α in the activation process of osteoclasts (Knowles and Athanasou 2009). Interestingly, when 24 h hypoxia/reoxygenation periods were repeated over an extended period in this study, enhanced osteoclast differentiation was detected, suggesting that diseased bone may present accelerated bone resorption via this mechanism. A previous study by the same authors also showed weak expression of HIF-2α in multinucleated, osteoclast-like giant cell tumor cells (Knowles and Athanasou 2008). However, the possible role of HIF-2α in osteoclasts remains to be elucidated.

2.4.4 Oxidative metabolism and hypoxia in cartilage

There are some conflicting reports about whether chondrocytes at the growth plate cartilage are hypoxic (Schipani et al. 2001; Shapiro et al. 1997). All in all, the usual consensus is that since growth plate cartilage is avascular, it is also at least partly hypoxic and, furthermore, hypoxia is in fact advantageous for chondrocyte development. Reflecting this consensus, a study by Amarilio and others (2007) showed that limb bud development in HIF-1α-deficient mice was retarded and that these mice displayed reduced cartilage formation. Reduced expression of Sox9, a key regulator of chondrocyte differentiation, was detected in these mice, linking hypoxia to the cartilage development process. As for articular cartilage, it has been detected to lack blood supply and therefore its matrix chondrocytes have been shown to experience a low ambient O_2 tension of appr. 1-6% (Zhou et al. 2004). Moreover, previous studies have shown that synovial fluid presents hypoxia with pO_2 levels at 7-10% in normal and diseased states (Lund-Olesen 1970). Some experiments have even shown that cartilage may survive in an anoxic environment for several days (Grimshaw and Mason 2000), indicating that the supply of oxygen needed for chondrocyte function is markedly lower than in most other cells.

It has become evident that O_2 tension is very important in modulating chondrocyte function. For instance, pO_2 has marked effects on chondrocyte phenotype and the expression of proinflammatory cytokines as well as type II collagen and aggrecan synthesis (Cernanec et al. 2002; Murphy and Polak 2004). Hypoxia has been observed to promote the differentiation of chondrocytes via a HIF-2α- and Sox9-dependent mechanism (Lafont et al. 2008) and, on the contrary, normoxia of 21% pO_2 may cause chondrocyte dedifferentiation and even decreased cell survival in cartilage (Schneider et al. 2007). Many *in vitro* experiments have shown that chondrocytes grow at significantly lower oxygen levels than most other cell types, and one of the main

causes for this is HIF. The *HIF-1α* knockout chondrocytes have been shown to be unable to maintain ATP levels at hypoxic microenvironments, indicating a requirement for this factor in chondrocyte metabolism (Pfander et al. 2003). Moreover, most pieces of evidence indicate that glycolytic metabolism is the primary source of energy in physiological conditions in chondrocytes, which further addresses the significance of low oxygen levels in cartilage physiology (Lee and Urban 1997; Marcus et al. 1973). In addition, the synthesis of VEGF in chondrocytes is induced by hypoxia, and this increase is lost in *HIF-1β* knockout cells (Pfander et al. 2003). An increasing number of studies suggest that the effects of low oxygen tension on cartilage are also mediated through ROS (Milner et al. 2007). Glycolytic metabolism prefered by chondrocytes causes a continuous flux of ROS in cartilage and, importantly, ROS may primarily act as physiological secondary messengers in chondrocytes. According to a hypothesis by Gibson and colleagues (2008), ROS as secondary messengers may lead to the activation of chondrocyte NHE that is involved in the transport of hydrogen and sodium, resulting in alterations in the hydrostatic pressure of cartilage matrix and cartilage matrix production by chondrocytes. According to these researchers, ROS may even have a more important role in chondrocyte metabolism than hypoxia itself, since they claim that oxygen would almost solely be needed in chondrocytes for ROS production. Supporting the role of ROS in chondrocyte metabolism, synoviocytes have in some studies been detected to release free radicals after cycles of anoxia/re-oxygenation (Schneider et al. 2005). Intriguingly, TGFβ has been shown to cause a pro-oxidative but not cytotoxic effect on articular cartilage by increasing ROS formation in chondrocytes, whereas IGF-1 has presented a possible antioxidant function in these cells (Jallali et al. 2007).

2.4.5 Significance of CA III in oxidative metabolism

Although mice lacking CA III are viable and fertile and have normal life spans (Kim et al. 2004), it is evident that CA III has important functions in cell physiology. CA III is especially abundant in type I oxidative muscle cells, whereas type II anaerobic muscle cells do not contain significant amounts of it (Väänänen et al. 1985). Thus, due to its localization in muscle cells, CA III has been linked with oxidative metabolism in the muscle (Barreiro and Hussain 2010). Exhaustive physical exercise enhances the production of free oxygen radicals due to hypoxia in muscle cells (Davies et al. 1982; Jenkins 1988; for review, see Thomas et al. 1995), and CA III has two cysteine residues which have been suggested to possess capacity for scavenging ROS. This argument is based on the finding that if exposed to hypoxia, CA III is capable of forming a disulfide link between glutathione and its two cysteine residues in S-glutathiolation (Chai et al. 1991). S-glutathiolation is one of the most important cellular responses to oxidative stress, aiming at the inhibition of irreversible protein oxidation (Cabiscol and Levine 1996). The hypothesis that CA III may be a ROS scavenger has further been supported by a study by Zimmerman et al. (2004) who found that the response of CA III to oxidative stress depends on the duration and severity of stress. CA III sulfhydryl oxidation due to mild oxidative stress is reversible and partial, whereas severe oxidative stress causes irreversible S-glutathiolation of both sulfhydryl groups in CA III protein, reflecting protein oxidation. In line with these results, Räisänen et al. (1999) showed that overexpression of CA III in NIH-3T3 cells may

protect the cells against H_2O_2-induced apoptosis because of the resulting reduced steady-state levels of intracellular ROS and increased cell proliferation rate. It has also been suggested that CA III might have a role in the regulation of apoptosis, since a study by Tang and others (2006) represented an upregulation of AKT and CA III expression in cells where the Brain and Reproductive Organ Expressed gene, encoding a conserved stress-modulating protein, was silenced by small interfering RNAs (siRNAs).

Additionally, CA III has been implicated in fatty acid metabolism. It has been shown that adipocyte cell lines express both CA II and CA III but only the amount of CA III is increased by the administration of insulin (Lynch et al. 1993). Moreover, leptin, a hormone secreted by adipose tissue, is capable of decreasing CA III expression (Alver et al. 2004). This suggests that CA III is needed in the oxidative metabolism of adipocytes. Interesting findings have also been made about the concentration of CA III in livers of adult male rats (Carter et al. 2001). The expression of CA III in male livers was approximately 30 times higher than in females and castration of male rats led to a reduction in CA III concentrations that could be partially restored by testosterone replacement. CA III expression in the liver has been detected to be predominantly localized in the perivenous areas of the liver (Carter et al. 2001). Moreover, dietary use of ethanol decreases CA III protein levels, linking the function of CA III to oxidative stress also in liver tissue (Parkkila et al. 1999). Furthermore, recent findings indicate that CA III levels in skeletal muscle cells of myasthenia gravis patients may be insufficient (Du et al. 2009).

A previous study claims that CA III antibodies may be present in the sera of patients with rheumatoid arthritis (RA), and that antigens for this antibody could be detected in the synovial membrane (Robert-Pachot et al. 2007). However, these new autoantibodies against CA III are not restricted to RA but are also found in other autoimmune diseases, such as systemic lupus erythematosus. Thus, further studies are needed to elucidate the significance of CA III antibodies in RA.

2.4.6 Metabolic bone disease in oxidative conditions

Coronary artery disease. Cardiovascular diseases have been associated with a number of bone pathologies, including Paget´s disease and renal osteodystrophy (Laroche and Delmotte 2005; Raggi et al. 2007; Schulz et al. 2004). The most consistent association, nevertheless, was demonstrated between osteoporosis and atherosclerosis, and particularly in relation to arterial mineralization (Hirose et al. 2003; Jorgensen et al. 2006; Magnus and Broussard 2005). Low bone mineral density has been shown to be associated with previous myocardial infarction, carotid atherosclerosis, arterial stiffness, and subsequent cardiovascular events (Hirose et al. 2003; Montalcini et al. 2004). This phenomenon may partly be explained by common risk factors of low bone mineral density and coronary artery disease, such as age, genetic associations, dyslipidemia, oxidative stress, inflammation, hyperhomocystinemia, hypertension, diabetes mellitus, and smoking. Additionally, there are examples of proteins that are important in regulating bone remodeling but that also have been implicated in

atherosclerosis, including osteocalcin, OPG, RANKL, osteopontin and BMPs (Johnson et al. 2006; Shao et al. 2006).

It has been speculated that coronary artery disease and osteoporosis may have a common molecular origin. A recent study by Almeida and colleagues (2009) showed that increased lipo-oxygenase-dependent lipid oxidation causes oxidative stress in bone. This activates the FoxO family of transcription factors and, in turn, attenuates β-catenin/TCF-mediated transcription, leading to depression of the *PPARγ* gene transcription (Almeida et al. 2009). The cascade ends with a decrease in Wnt signaling, and since canonical Wnt signaling is necessary for osteoblast differentiation and survival (for review, see Roblig and Turner 2009), coronary artery disease may lead to decreased bone mineral density. This mechanism may partly explain the high incidence of osteoporosis in these patients. Interesting suggestions have also been made about the role of bone marrow stem cells, which can give rise to endothelial and vascular smooth muscle cells (Caplice and Doyle 2005). Circulating osteoblast progenitor cells may play a role in the calcification of blood vessels and heart valves, an active process detected as a common complication of atherosclerosis (Abedin et al. 2004). In fact, increasing evidence shows that a process similar to endochondral ossification may occur in diseased heart valves, causing the calcification process (Caira et al. 2006). Intriguingly, both BMP 4 and RANKL expressions have recently been connected with calcification in vascular smooth muscle cell populations (Mikhaylova et al. 2007; Panizo et al. 2009). Another interesting link of coronary artery disease to bone was described by Matsubara and colleagues (2009) who found that patients with cardiovascular disease have elevated blood OPG levels, which in turn is associated with inflammation and oxidative stress. Furthermore, patients with high OPG presented increased cardiovascular mortality. Therefore, it is clear that there is a close relationship between atherosclerosis and bone, and the mechanisms underlying this association appear to include the involvement of bone marrow-derived cells in the repair and pathology of arteries, and oxidative stress.

A single missense mutation in LRP6, a coreceptor for the Wnt-signaling pathway, has been genetically linked with diabetes and osteoporosis, as well as early coronary artery disease, hyperlipidemia and hypertension (Mani et al. 2007). Hence, it is not surprising that type 1 diabetes has previously been strongly associated with serious skeletal disturbances, including decreased linear bone growth during pubertal growth (Ahmed et al. 1998; Salerno et al. 1997), osteopenia and osteoporosis (Hampson et al. 1998; Kemink et al. 2000), an increased risk of fragility fracture (Janghorbani et al. 2006) and poor bone healing and regeneration following injury (Loder 1988). Analysis of serum markers for bone remodeling in animals with type 1 diabetes suggests that bone resorption is unaltered (Bonfanti et al. 1997; Kemink et al. 2000) or even decreased (Gunczler et al. 1998). Thus, bone pathology in type 1 diabetes results from deficits in bone formation that occur in both spontaneous and induced models of diabetes (Lu et al. 2003; McCabe 2007; Thrailkill et al. 2005a). Several genes regulating osteoblastogenesis and chondrogenesis, such as *Runx2*, are downregulated due to diabetes (Fowlkes et al. 2007). Interestingly, systemic insulin administration markedly improves bone formation in diabetic animals (Thrailkill et al. 2005b), and even local

delivery of insulin to a fracture site has been suggested to stimulate bone regeneration in diabetes (Thrailkill et al. 2005b). Recent studies in this area indicate that ROS may play an important role in the initiation of both diabetes and osteoporosis, and activation of the FoxO pathway and antagonism of Wnt signaling seem to be the common molecular factors in both conditions (for review, see Manolagas 2010). However, although it is already known that there exists a correlation between increased fracture risk and type 2 diabetes, the influence of oxidative stress on this relationship is not currently known.

3 AIMS OF THE STUDY

Regulation of oxidative metabolism and acid-base balance in calcified tissues are fairly unknown subjects even though diseases in calcified tissues can be directly associated with changes in these systems. Therefore, we explored the role of carbonic anhydrases and bicarbonate transport proteins in bone resorbing human and murine osteoclasts. We also studied oxidative metabolism at the murine cartilage-bone interface where calcification occurs.

Thus, the specific aims of this study were to:

- Identify and localize possible bicarbonate transport metabolon proteins in osteoclasts, including membrane-bound CA isoenzymes IV, IX, XII and XIV and an electroneutral sodium-dependent bicarbonate co-transport protein NBCn1;

- Assess the significance of CA IV and CA XIV as well as NBCn1 in bone resorption by osteoclasts *in vitro*;

- Evaluate the physiological role of CA III in chondrocyte metabolism at the murine bone-cartilage interface.

4 MATERIALS AND METHODS

4.1 Immunohistochemistry (I-III)

4.1.1 Preparing rat bones for immunohistochemistry (I-II)

Perfusion fixation of rat bones was used to allow antigen preservation and excellent tissue morphology. Three-day-old rat pups were heparinized to avoid clotting and then anesthetized according to routine procedures. A cannula was inserted to the left ventricle for vascular delivery of the fixative and a cut was made in the right atrium. PBS (phosphate-buffered saline) was first injected through the cannula with a peristaltic pump (1 ml/min), following a vasodilator flush with 0.2% ethyl acetimidate in PBS. After this, 4% paraformaldehyde – 0.25% sucrose in 0.2 M phosphate buffer, pH 7.2) was injected for 5-12 minutes at 1.5 ml/min. Immediately after perfusion, femori, tibiae and humeri were dissected and immersion fixed in paraformaldehyde overnight. Bone samples were washed in PBS for 30 min and decalcified in 14% EDTA (pH 7.2). The bones were finally embedded in paraffin and cut into three- or five-µm sections with a microtome.

4.1.2 Dissection of embryonal mouse cartilage (III)

Female non-transgenic C57b1 x DBA Del 1 mice were used in study III. The gender of the animals was determined at embryonal stage by amplifying the Sex-Determining Region Y (primers: 1) agtgttcagccctacagc, 2) gagtacaggtgtgcagct). Mouse knee joints were dissected under microscope to obtain epiphyseal cartilage from embryonal days E 16.5, E 18.5, E 20.5, and postpartum days D 0 and D 20, as well as 2 and 6 months after birth. For histology, the samples comprising the entire knee joints were dissected at the levels of femoral and tibial growth plates, fixed, mounted and cut with a microtome into three- or five-µm sections. For RNA isolation and subsequent qPCR, only epiphyseal cartilage was collected under a microscope.

4.1.3 Antibodies (I-III)

Polyclonal rabbit anti-CA IV and anti-CA XII antibodies have been characterized and described previously (Tureci et al. 1998; Zhu and Sly 1990). Also the monoclonal anti-CA IX antibody was characterized before (Pastorekova et al. 1997). The polyclonal anti-CA XIV antibody (Parkkila et al. 2002) and polyclonal anti-NBCn1 antibody (Vorum et al. 2000), as well as the in-house polyclonal antibodies against TRAcP (Alatalo et al. 2000) and CA III (Räisänen et al. 1999) were used as described before.

4.1.4 Immunohistochemical analysis of rat and mouse bones (I-III)

Immunostaining procedures were performed according to the following protocol: Rehydrated bone specimens were permeabilized with ficin solution or hyaluronidase solution (both from Zymed), and non-specific antibody reactions were blocked with 3% bovine serum albumin (BSA). Endogenous peroxidase activity was blocked with

3% H_2O_2 treatment, and endogenous biotin binding sites were blocked with an Avidin-Biotin blocking kit, as suggested by the vendor (Vector Laboratories). CA IV, CA XII and CA XIV antibodies were used at 1:3000 dilutions, CA IX was diluted 1:10, and the CA III antibody was used at 1:2000 dilution. NBCn1 antibody was used at 1:300 dilution. After an overnight incubation at +4°C, bone specimens were rinsed with PBS, and streptavidin-conjugated, species-specific secondary antibodies were added (Vector Laboratories, diluted 1:300 in 0.5% BSA), followed by detection with ABC and DAB reactions (Standard ABC Elite and Peroxidase Substrate Kits, Vector Laboratories). Nuclei were visualized by methyl green counterstaining (Vector Laboratories) and samples were mounted in Histomount.

4.2 Cell and tissue culture methods (I-II)

4.2.1 Rat osteoclast cultures (I-II, unpublished)

Osteoclasts were mechanically harvested from newborn rat long bones and cultured on bovine cortical bone slices as described before (Lakkakorpi et al. 1989). After 30 min, unattached cells were rinsed away and cells were cultured in α-MEM pH 7.0 containing 20 mM HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (Invitrogen). The culture atmosphere consisted of 5% CO_2/ 95% air at 37°C. For osteoclast activation experiments, human RANKL (Peprotech) was added into the culture media to a final 20 ng/ml concentration after an incubation period for cell attachment. For experiments studying rat osteoclast NBCn1 mRNA expression in different extracellular pH conditions, pH of the culture media was set to 6.6 or 6.8 with hydrochloric acid or to 7.6 with sodium hydroxide immediately before culturing. The cells were then divided into three groups: exposure to normal (pH 7.2), alkaline (pH 7.6) or acidic (pH 6.6 or 6.8) culture medium. After 4, 24 or 48 hours, cultures were either stopped by fixing the cells with 3% paraformaldehyde - 2% sucrose or, in case of RNA extraction, bone slices were transferred into an RNA extraction buffer.

4.2.2 Human osteoclast cultures (I-II)

The study protocol and use of human material were approved by the Ethics Committee of the Hospital District of Southwest Finland (Turku, Finland). Venous blood samples were drawn from healthy adult male volunteers into heparin tubes (Venoject, Terumo) and CD14-positive cells were isolated as described before (Husheem et al. 2005). A total of 10^6 CD14+ cells were cultured either on glass coverslips or on bovine cortical bone slices for 3 or 7 days. Cells were cultured in α-MEM (pH 7.0) supplemented with 20 mM HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (Invitrogen). To induce osteoclast formation, 20 ng/ml of soluble human RANKL (Peprotech) and 10 ng/ml of human M-CSF (R&D Systems) were added to the culture medium. At the desired time points, samples were either fixed in 3% paraformaldehyde or transferred into RNA extraction buffer (GenElute Mammalian total RNA isolation kit, Sigma Chemical Co). As for human osteoclast studies in article I, human osteoclast precursor cells were purchased from Lonza and cultured for 7 days in the presence of M-CSF, RANKL and TGF-β1 as suggested by the vendor.

4.2.3 Human osteoblast cultures (II)

Human mesenchymal stem cells were purchased from Lonza, and osteogenic induction was carried out according to the manufacturer´s instructions. All inductions were performed on 48-well plastic plates in 0.5 ml of culture medium (Lonza). A total of 20 000 cells were added to each well and the cells were cultured for 24 hours in the presence of 5μg/ml of polybrene to prepare the cells for lentiviral transfection. The cells were transduced with lentiviral particles as described below, and osteoblast differentiation was allowed to continue for 4 or 8 days. For end-point assays on days 4 and 8, cells used for histological evaluation were fixed in 3% paraformaldehyde, and cells used for ALP activity measurements were lysed in 50 mM Tris-HCl (pH 7.6), 0.1% Triton X-100, 0.9% NaCl. ALP activity measurements were performed as described before (Heino et al. 2004), and another set of fixed cells was stained for ALP (Leukocyte alkaline phosphatase kit, Sigma Aldrich) prior to microscopical evaluation.

4.2.4. Fetal mouse metacarpal cultures (III)

To study CA III expression in embryonal cartilage, fetal mouse metacarpi were cultured *in vitro* as described before (Laitala-Leinonen et al. 1999). Embryos were collected at the age of e15.5, when hypertrophic chondrocytes are not yet present in the cartilage anlage. Metacarpals were isolated by microdissection and cultured for 7 days in α-MEM supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.1% BSA (Invitrogen) under either normoxia, hyperoxia (pO_2 30%) or hypoxia (pO_2 2%). Samples were then fixed in 4% neutral formalin and decalcified in formic acid. Decalcified samples were embedded in paraffin, serial sections were cut using a microtome and Hematoxylin-Eosin (HE) staining was performed for morphological analysis. Alternatively, total RNA was extracted from the metacarpals after 7 days of culturing and used for PCR as described below.

4.3 Cytochemistry, microscopy and shRNA experiments

4.3.1 Immunocytochemistry, cytochemistry and microscopy (I-III)

Before staining, fixed cells were permeabilized with 0.5% Triton-X 100 for 4 minutes. Cells were then histochemically stained for TRAcP (Leucocyte acid phosphatase staining kit, Sigma Aldrich). Alternatively, cells were stained for cathepsin K using a monoclonal antibody (Acris) or for NBCn1 using polyclonal NBCn1 antibodies described before. ER and Golgi markers calnexin (Abcam) and AlexaFluor^{488} HPA (Helix pomatia agglutinin, Invitrogen) were used for article II together with NBCn1 antibodies. Rhodamine-labeled secondary antibodies were purchased from Jackson Immunochemicals. For actin ring visualization, fixed cells were incubated for 20 min in AlexaFluor^{488}- or ^{568}Phalloidin (Invitrogen). For detection of nuclei, Hoechst or DAPI (both from Invitrogen) was used as a counterstain and samples were mounted in either Vectashield (Vector Laboratories) or the Prolong Gold Anti-Fade Reagent (Invitrogen). The number of osteoclasts was counted under a fluorescence microscope. Cells with at least 3 or more nuclei and actin rings were regarded as osteoclasts in our studies. In article II, TRAcP- or cathepsin K-positive cells with nuclei counts of 3 or more were regarded as osteoclasts. Rhodamine-conjugated WGA lectin (Whear Germ

Agglutinin, Sigma Aldrich) was used to visualize resorption pits in osteoclast cultures as described before (Selander et al. 1994).

Confocal microscopy was performed with a Leica TCS-SP confocal laser scanning microscope equipped with an Argon-Krypton laser and LCS Leica Confocal Software version 2.0 (Leica Microsystems GmbH). Alternatively, samples were analyzed using a Leica DM IRB inverted microscope equipped with a CSU-10 laser confocal scanning unit (Yokokawa, Tokyo), an Argon-Krypton laser (Melles Griot) and a CoolSNAP HQ camera (Photometrics). Fluorescent images were acquired by using Metamorph software (Molecular Devices Corporation).

4.3.2 Lentiviral NBCn1 shRNA transduction (II)

Transduction-ready lentiviral particles containing a pool of three *SLC4A7*-specific shRNAs were purchased from Santa Cruz Biotechnology, Inc. For control transductions, negative scrambled shRNA–containing lentiviral particles were purchased and used. Another non-transduced control was included in all assays to evaluate possible toxicity of the lentiviral particles. For NBCn1 shRNA transductions, a total of 20 000 human CD14+ cells were seeded onto bovine cortical bone slices in 24 well plates, and the cells were cultured in 1 ml of culture media containing RANKL and M-CSF for 6 days. To improve transduction efficiency, 5 μg/ml of polybrene (Millipore) was added to the culture medium 24 h before transduction. Lentiviral particles were diluted in fresh cell type-specific differentiation medium containing 5 μg/ml of polybrene. The culture medium on the 24-well plates was replaced with 200 μl of shRNA lentivirus dilution, and cells were further cultured for 2-8 days. Cell viability was monitored microscopically after Hoechst staining and by using the Cell Titer Blue assay (Promega). Intracellular acidification was studied by acridine orange incorporation studies as described before (Palokangas et al. 1997). Bone resorption activity in these samples was evaluated by measuring the release of C-terminal fragments of type I collagen from the culture medium by Serum Crosslaps ELISA (IDS).

4.4 RNA extraction and polymerase chain reaction methods (I-III)

4.4.1 RNA extraction and quantitative/real-time PCR (I-II)

After culturing rat osteoclasts on bone slices for the necessary time periods, RNA was extracted from the mixed cell population using GenElute mammalian total RNA kit (Sigma, USA), and genomic DNA was removed with RNase-free DNAse1 (Ambion, USA) (I-II). Alternatively, Trizol reagent (Invitrogen) was used for RNA extraction according to the manufacturer´s suggestions (unpublished). RT-PCR reagents were purchased from Finnzymes (Finland) (I) or Promega (USA) (unpublished). Rat CA-specific PCR primers were designed using the following Biology Workbench Primer3 program (http://workbench.sdsc.edu/) being as follows: CA IV forward TGG TTG AGG TGG GAA ACG-3´and reverse 5´-TGA GCC CTG GTA ACG GAA´. CA IX forward ACC TCA GTA CTG CTT T-3´and reverse TTC CAA ATG GGA CAG CAA. CA XII forward TTG AAC CTA ACC AAT GAT GGC and reverse GAA CAG CAA GGA CAG CGA G (I). Since the rat *CA XIV* gene sequence was not available

during our studies, CA XIV primers were designed by comparing human and mouse CA XIV mRNA sequences and using conserved regions of the sequence in primer design, the outcome being as follows: CA XIV forward TCA ACA GTG AG CCA C and reverse TGT GAG TGA GCC GTT GTA GC (I). The total RNA extracted from a 2-day old rat kidney was used as a positive control (I, unpublished).

In study I, ten nanograms of total RNA was used as templates for reverse transcription at +42°C for 50 min, followed by denaturation at +94°C for 2 minutes. The PCR cycling protocol comprised 31 cycles of denaturation at +94°C for 30 sec, annealing at primer-specific temperatures (+56°C for CA IV; +48°C for CA IX; +60°C for CA XII and +56°C for CA XIV) for 30 sec and extension at +72°C for 60 sec. To evaluate the differences in expression levels between CA XIV and GAPDH, samples were removed from the PCR machine after 15, 20, 25 and 30 cycles for further analysis in 3% agarose gels.

In study II, quantitative RT-PCR was performed as described before (Hu et al. 2005) using the Opticon DNA Engine (MJ Research, USA) and DyNAmo SYBR Green 2-step qRT-PCR system (Finnzymes, Finland). A 66nt long sequence of human NBCn1 (*SLC4A7*) was amplified using primers ACC CTC ACT TG CTT GAA AGG (forward) and GAC CTG TTC GCA AAG AGT GG (reverse). GAPDH was amplified as the housekeeping gene (Hu et al. 2005). NBCn1 fluorescence was normalized with the GAPDH reference gene fluorescence, and fold change in NBCn1 gene expression was calculated using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen 2001).

In unpublished RT-PCR experiments measuring NBCn1 mRNA changes in rat osteoclasts after exposure to anoxia or after alterations in extracellular pH, the NBCn1, NHE 1 and CA II primers were used as follows: NBCn1 forward GCA TTT GTG AGA CTG GCT CCT GCA GTT CTC and reverse TCC TAA GTG AAA GAG TTT CTC CAA GGC TTC (Choi et al. 2000). NHE 1 forward CCT TTC TGG GGT TTA CAC GGG AGG GAC TGT3 and reverse GTG GAG CTC TGA CTG GCA GGG AAG ATT (Lee et al. 1998). CA II forward TGT GCA GCA CCC AGA TGG and reverse CAG TTC TTC AGC CTC CCC (Hermo et al. 2005). GAPDH housekeeping gene primers were used as a control as described before (Hu et al. 2005). The PCR protocol was as following: two to four nanograms of total RNA was used as template for reverse transcription at 45°C for 45 min, followed by denaturation at 94°C for 2 min. The PCR cycling protocol comprised 35 cycles of denaturation at 94°C for 30 s, annealing at primer-specific temperatures (62°C for electroneutral NBCn1, 62°C for GAPDH, 60°C for CA II and 71°C for NHE1) for 1 min, and extension at 68°C for 2 min. Final extension was performed for 7 minutes at 68°C. Amplification products were size-fractionated in 2.5% agarose gels and visualized with ethidium bromide. Each PCR was repeated at least three times and samples were analyzed densitometrically. The relative expression of NBCn1 mRNA was counted by dividing the relative optical density of the specific product by GAPDH intensity in the same sample.

4.4.2 RNA extraction from cartilage and real-time PCR (III)

In study III, cDNA synthesis, Affymetrix cDNA microarray analysis and qPCR were performed at the Finnish Microarray and Sequencing Centre (Turku Centre for Biotechnology, Finland). Total RNA was extracted from the metacarpus cultures after 7 days of culturing, and from the embryonal mouse knee epiphyseal cartilage on days e18.5, e20.5 and 5 days and 1 month after birth, and from femoral muscle on day 5 after birth, by using a modification of the guanidium isothiocyanate method (Chirgwin et al. 1979). The samples comprising the entire knee joints were microdissected at the levels of femoral and tibial growth plates. Before extraction, skin and muscle tissues were carefully removed from the knee joints. (Säämänen et al. 2007). Dissected tissues were pulverized under liquid nitrogen prior to RNA extraction. After extraction, 1 μg of total RNA was treated with RQ1 RNAse-free Dnase (Promega) to remove genomic DNA. The first strand cDNA was synthesized with the M-MLV Reverse Transcriptase RNase H Minus product according to the manufacturer´s instructions using oligo (dT)15 primer (all from Promega). Real-time PCR was performed with the ABI PRISM 7900HT Sequence Detection system (Applied Biosystems). Total reaction volume was 10 μl, containing 2 × ABsolute QPCR ROX Mix (ABgene), 2 ng/μl of cDNA, sense and antisense primers in 400 nM concentration (for CA III, forward: 5´-AGGTGACCATGGCTAAGGAG-3´ and reverse: 5´-TTCATGCCAGTGA TCAGGAC-3´; for GAPDH forward: 5´-TGCACCACCAACTGCTTAG-3´ and reverse: 5´-GGATGCAGGGATGATGTTC-3´), and Universal ProbeLibrary Probe (#17 for CA III 5´-CAGCCACA-3´ and 5´-CAGAAGACTGT GGATGGCCCCTC-3´ for the housekeeping gene GAPDH; Roche Applied Science) at 100 nM concentration. Muscle mRNA samples obtained on day d5 after birth were used as a control. CA III expression was normalized using GADPH as an internal standard. A relative CA III expression value for each sample was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All qPCR analyses were performed twice for verification of the Affymetrix cDNA microarray data.

4.5 Carbonic anhydrase inhibitor studies (I)

4.5.1 Effects of PCS on primary rat osteoclast cultures (I)

To examine the effects of CA inhibition, primary rat osteoclasts were cultured in the presence of 1 μM or 10 μM acetazolamide or in the presence of 1 μM, 10 μM or 100 μM membrane-impermeable carbonic anhydrase inhibitor PCS. PCS is a potent CA inhibitor for both cytosolic and membrane-bound CAs and it is membrane-impermeant due to its permanent positive charge at the pyridinium ring, which interferes with its crossing of biological lipid membranes (Casey et al. 2004). Acetazolamide also shows very good inhibitory activity against cytosolic and membrane-associated CAs. Inhibitors were added to the culture medium at the beginning of the culture after the attachment period of osteoclasts on bone slices. Addition of PCS or acetazolamide to cell culture medium did not affect the pH of the culture media. After 48 hours, cells were fixed in 3% paraformaldehyde - 2% sucrose, and Phalloidin and Hoechst stainings were performed as described above. Samples were examined with a fluorescence microscope and the numbers of osteoclasts and actin rings were counted.

After this, cells were removed from the bone slices and WGA-lectin staining was performed to visualize resorption pits as described before (Selander et al. 1994).

4.5.2 Cell viability after PCS treatment (I)

In order to evaluate the possible effects of PCS and acetazolamide on cell viability, primary rat osteoclasts were plated on 96 well plates. Unattached cells were rinsed away after a 45-min attachment period and 1 μM, 10 μM or 100 μM PCS or 10 μM acetazolamide was added. For system validation, 0.25% sodium azide was added to some wells. After 48 h, Live/Dead viability assay (Molecular Probes Invitrogen, USA) was used to evaluate the amounts of live and dead cells. Dead-to-live cell ratio was determined by dividing the fluorescence obtained from Ethidium homodimer measurement (indicates dead cells) by the fluorescence obtained from Calcein AM measurement (indicates live cells). Measurements from 2 separate experiments with 3 replicas each were performed, and relative dead-to-live cell ratio in PCS-, acetazolamide- or sodium azide-treated samples were calculated as percentage of the dead-to-live cell ratio observed in the baseline samples.

4.5.3 Detection of intracellular pH after PCS treatment (I)

Intracellular acidification in rat osteoclasts was studied by staining live cells with acridine orange (Hu et al. 2005). Primary rat osteoclasts were cultured for 48 hours in several concentrations of acetazolamide or PCS. Live cells were then incubated in 5 μM acridine orange (Sigma, USA) for 15 min, after which samples were quickly rinsed with fresh medium. Cells were immediately viewed under a fluorescence microscope and the amount of intracellular acidification was evaluated.

4.5.4 Exposure of rat osteoclasts to vitamin D and CA inhibitors (unpublished)

Primary rat osteoclasts were harvested and cultured as described above. After the cell attachment period, the cultures were incubated in a standard culture media with 1 μM, 10 μM or 100 μM PCS or 10 μM acetazolamide. Moreover, vitamin D ($1,25(OH)_2D_3$) was added into the cultures in the final 10^{-8} M concentration. After 2 days of culturing, the samples were fixed and the cells were stained for Phalloidin and Hoechst as described above. The samples were viewed under microscope and the number of actin rings was counted.

4.6 Hypoxic and anoxic experiments (II, III)

4.6.1 Rat osteoclasts as subjects to hypoxia (unpublished)

Primary rat osteoclast cultures were prepared as described above. After a 30-minute attachment period to bone surface in normal culture conditions, the cells were incubated in standard culture media with 1 μM, 10 μM or 100 μM PCS or 10 μM acetazolamide. Some of the cultures were transferred to a hypoxic chamber, whereas a part of the cultures remained in normal conditions for control. For hypoxic cultures, an environment with 1% oxygen, 5% CO_2 and 94% N_2, +37°C, was maintained during 3 days of culture, whereafter cells were fixed inside the hypoxic chamber to avoid redox

reactions. The cells were stained with Phalloidin and Hoechst and visualized in order to count the numbers of multinuclear cells and actin rings. Moreover, hypoxic and normoxic cultures were stained for TRACP and positive cells were counted under a microscope. In addition, a TRACP immunoassay was performed as described earlier (Alatalo et al. 2000): TRACP antiserum was added onto anti-rabbit IgG-coated microtiter plates (EG & G Wallac) for 1 h. Cell culture media (200 µL) from each sample group were incubated in the wells for 1 h, and bound enzyme activity was detected using 8 mmol/L 4-nitrophenyl phosphate as substrate in 0.1 mol/L sodium acetate buffer for 2 h at 37°C. The enzyme reactions were terminated by adding 25 µL of 0.32 mol/L NaOH to the wells, and the absorbance at 405 nm (A_{405}) was measured with a model 2 Victor instrument (EG & G Wallac).

4.6.2 Rat osteoclasts as subjects to anoxia (unpublished)

Primary rat osteoclasts were harvested and pre-cultured on bovine bone slices in normoxia for 24 hours as described above. An anoxic incubator (anaerobic system model 1024, Forma Scientific; 10% H_2, 5% CO_2 and 85% N_2, 37°C) was used to study changes in NBCn1 mRNA expression after exposing the cells to anoxia for 5 min or 20 min. Trizol reagent and paraformaldehyde solution were balanced on ice in the anoxic incubator before use, and the RNA extraction as well as cell fixation procedures were initiated inside the chamber to avoid redox reactions.

4.6.3 Detection of ROS in cartilage samples (III)

Long bones of mouse embryos were dissected and immediately embedded in liquid nitrogen to snap-freeze the samples. The samples were cut into 5-micrometer sections with a cryotome and stored at -20°C before use. Carboxy-H2DCFDA (5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate) was purchased from Molecular Probes, USA, and prepared according to the manufacturer´s instructions. Immediately before use, Carboxy-H2DCFDA was diluted with a phosphate buffer (pH 7.0) down to a 50 µM concentration. Hoechst nuclear stain was added into the solution for 1:600 final concentration. Samples were incubated in the solution for 30 min at +37°C in the dark. After washing, immersion fixation of the samples was performed in 3% paraformaldehyde. The samples were mounted in Vectashield and visualized immediately.

4.6.4 Oxidative challenge in fetal mouse metacarpal cultures (III)

To provoke possible changes in CA III expression, metacarpal cartilage anlage isolated from e16 mouse embryos were cultured at hypoxia (1% O_2), normoxia or hyperoxia (30% O_2) for 6 days as described before. Fixatives were allowed to reach equilibrium at the desired oxygen level before adding them to the samples, and samples were removed from the hypoxia or hyperoxia chambers only after fixation. Fixed samples were embedded in paraffin and sectioned for subsequent immunohistochemistry using a microtome.

4.7 *In vivo* analysis (II)

4.7.1 An experimental animal model of systemic metabolic acidosis

Seven-week-old Sprague-Dawley rats (200-250 g) were assigned randomly to either control group or NH_4Cl-treated group. To examine the effects of chronic metabolic acidosis on osteoclast NBCn1 protein expression, the rats were treated orally with 0.28 M NH_4Cl in the drinking tap water for 2 weeks *ad libitum* ($n = 5$). Control rats received tap water *ad libitum* ($n = 5$) and all rats had free access to standard rodent food. During the last two days of the experiment, the rats were kept in metabolic cages to collect urine on each morning. The pH of urine collected for the last 24 h before termination of the experiment was measured with a PHM83 pH meter (Radiometer, Copenhagen, Denmark), and urine osmolality was measured with an automatic cryoscopic osmometer (Omomat 030, Gonotech, Berlin, Germany). Venous blood was drawn in gas-tight syringes from the inferior vena cava. Blood was centrifuged for 15 min at 4000 *g* to remove blood cells, and the plasma was analyzed for sodium, potassium, blood urea nitrogen (BUN) and creatinine with a Vitros 950 (Johnson & Johnson, Table 1, II).

4.8 Promoter analysis

4.8.1 Promoter analysis of NBCn1 (SLC4A7)(unpublished)

To gain more insight into the physiological role of NBCn1 in resorbing osteoclasts, potential transcriptional regulators were studied by *in silico* promoter analysis (unpublished). Since annotation of the true 5' end of many genes is still uncertain, we used a CAGE tag database (Kawaji et al. 2006) to locate transcription start sites (TSSs) in mouse and human *SLC4A7*, the gene encoding NBCn1. The UCSC genome browser was used to view highly conserved regulatory regions (Karolchik et al. 2003) in the close vicinity of *SLC4A7*. 1.5kb sequences neighboring human (genome version hg_18) and mouse (genome version mm_8) *SLC4A7* potential TSS were retrieved from USCS sequence database. To predict transcription factor binding sites (TFBSs), we employed Conreal (Berezikov et al. 2006) with high quality matrices from TRANSFAC professional 9.4 (Matys et al. 2003, Wingender et al. 1997). Ingenuity Pathways Analysis version 5.0 (Ingenuity® Systems, www.ingenuity.com) was used to study the interactions of TFs with binding sites in the *SLC4A7* promoter region. The TFs were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base, and networks were then algorithmically generated based on their connectivity. Fischer's exact test was used to calculate a p-value determining the probability that the association between the genes in the interaction pathway is explained by chance alone. Graphical representations of the molecular relationships between genes / gene products were also produced. Genes / gene products were represented as nodes, and the biological relationship between two nodes was represented as an edge (line). All edges are supported by at least 1 reference from the literature. Nodes were displayed using various shapes that represent the functional class of the gene product. This analysis was carried out by Anne Seppänen, MSc.

4.9 Statistical analysis (I-III)

All values are presented as means ± standard deviation. Data were analyzed by unpaired t-test between the two groups or by one-way analysis of variance (ANOVA) followed by multiple comparisons test when the number of study groups was more than two. Multiple comparisons tests were only applied when a significant difference was determined in the ANOVA, $P<0.05$. P values < 0.05 were considered significant. In article III, the mRNA sample groups consisted of epiphyseal cartilage samples from embryonal days E18.5 (n=3), E20.5 (n=4), and epiphyseal cartilage samples from postnatal days D5 (n=4), and D30 (n=2) after birth. In addition, muscle mRNA samples (n=4) obtained on D5 were used as a control.

5 RESULTS

5.1 Membrane-bound carbonic anhydrases in rat osteoclasts (I)

5.1.1 Expression of membrane-bound CAs in bone

To define the expression of membrane-bound carbonic anhydrase isoenzymes CA IV, CA IX, CA XII and CA XIV in rat bone, we performed immunohistochemical stainings on 3-day-old rat bone specimens. Our results showed that weak CA IV staining was observable in a small population of bone marrow cells, in cells lining the bone trabeculae, and in hypertrophic cartilage chondrocytes, while osteoclasts remained negative for CA IV (I, Fig. 2 b). CA IX staining of bone was mostly negative, with some faint staining present only in a population of bone marrow cells and more intense staining in the hypertrophic cartilage (I, Fig. 2 c). In turn, CA XII staining was quite intense, since many bone marrow cells, most of the bone lining cells and chondrocytes were stained with the CA XII antibody (I, Fig. 2 d). Positively stained tissue macrophages were also detectable in bone marrow, but osteoclasts remained negative. CA XIV staining in bone was very intense, with multiple CA XIV-positive bone marrow cells (I, Fig. 2 e-f). Osteoclasts showed an intense positive CA XIV reaction on their basolateral plasma membrane and, to a lesser extent, in their cytoplasm. In the positively stained mononuclear bone marrow cells, both nuclei and plasma membranes were stained with the CA XIV antibody. Moreover, both proliferative chondrocytes and hypertrophic chondrocytes expressed CA XIV. Thus, we concluded that CA XIV was the only membrane-bound carbonic anhydrase expressed at protein level in rat osteoclasts.

We also used confocal microscopy to define the subcellular localization membrane-bound CA isoenzymes in human osteoclasts, and observed a distinct plasma membrane staining for both CA IV and CA XIV in non-permeabilized human osteoclasts. In permeabilized cells, some punctuate CA IV staining was visible inside the osteoclasts near to the sealing zone (I, Fig. 3). Moreover, strong staining in perinuclear vesicles was observed with the CA XIV antibody in permeabilized cells (I, Fig. 3). This positive reaction was specifically located in the uptake zone of osteoclastic degradation products.

Finally, we performed RT-PCR on RNA extracted from 2-day old primary rat osteoclast cultures to confirm the expression of CA isoenzymes in these cells (I, Fig. 4). GAPDH was used as a housekeeping gene control in this experiment. We detected CA IV and CA XII and CA XIV mRNA in cultured rat osteoclasts, but CA IX mRNA expression was not observable. GAPDH mRNA expression significantly exceeded CA XIV mRNA expression in the primary rat osteoclast cultures, however.

5.1.2 Effects of membrane-bound CA inhibition on osteoclasts

After detecting the expression of several membrane-bound carbonic anhydrase isoenzymes in rat osteoclasts, we wanted to explore the significance of these enzymes in bone resorption. Therefore, we used a membrane-impermeable sulfonamide CA inhibitor PCS in our experiments. We cultured primary rat osteoclasts in the presence of PCS or acetazolamide, a soluble CA inhibitor, for two days, and monitored possible changes in cell and nuclear morphology after the treatment.

We observed several changes in osteoclast morphology and function due to the inhibition of membrane-bound carbonic anhydrases. Our control cultures refering to acetazolamide-treated cultures and cultures with baseline culture media, displayed no major differences as regards osteoclast and actin ring numbers. However, PCS-treated cultures showed remarkable changes: there was an increase in the numbers of osteoclasts and actin rings in cultures treated with 1 μM PCS compared to other cultures (I, Fig. 5). In contrast, a dose-dependent decline in osteoclast survival was seen due to 10 and 100 μM PCS treatment, and the number of multinuclear osteoclasts was significantly lower in cultures containing these amounts of PCS as compared to the baseline (I, Fig. 5). Furthermore, the number of actin rings after exposure to 10 and 100 μM PCS was lower than the number of actin rings in baseline cultures. Thus, we drew the conclusion that PCS has a dose-dependent effect on osteoclast survival and function, and possible toxic effects due to higher levels of PCS lead to osteoclast apoptosis and loss of attachment to bone, reflected by disturbed actin rings (I, Fig. 6). In addition to monitoring actin rings, we used phase contrast microscopy to visualize the actual resorption capacity of PCS-treated osteoclasts. We saw that cells with disrupted actin rings were often located on a resorption lacuna, suggesting that the initiation of the bone resorption process in these cells had been normal. However, as we visualized the resorption pits with WGA lectin attaching to the proteoglycans in the resorption lacuna, we saw a significant decrease in the total number of resorption pits in cultures subjected to PCS treatment (I, Fig. 7). Interestingly, long-term treatment with acetazolamide did not have such a deleterious effect on bone resorption.

In addition, we used acridine orange to detect the state of intracellular acidification in rat osteoclasts after membrane-bound carbonic anhydrase inhibition. In this experiment, we observed that actively resorbing osteoclasts were near neutral with only a few acidic vesicles scattered within the cytoplasm (I, Fig. 9), since these cells had already secreted most of their acidic vesicles into the resorption lacuna. In contrast, osteoclasts cultured in the presence of acetazolamide showed numerous acidic vesicles. The effect of PCS depended on inhibitor concentration. When osteoclasts treated with 100 μM PCS were studied, an intense acidic vesicle staining was seen in the cytoplasm (I, Fig. 9). The accumulation of acidic vesicles gradually diminished in samples treated with 10 μM and 1 μM PCS, so that osteoclasts cultured in 1 μM PCS contained only few acidic vesicles close to their cell membrane. This observation is interesting as it appears that intracellular lysosomal acidification, essential to bone resorption, remained functional in both PCS- and acetazolamide-treated cells but the treated cells were not able to extrude the vesicles.

We decided to study the net effect of PCS on cell viability in primary osteoclast cultures. Thus, we performed Live/Dead stainings on rat osteoclast cultures exposed to PCS. We were able to observe a very high ratio of cell death in cultures treated with 100 µM PCS and, thus, we confirmed that high concentrations of membrane-impermeable CA inhibitor were able to launch either cell apoptosis or necrosis in our cultures (I, Fig. 8). On the other hand, 10 µM PCS enhanced the cell survival in comparison with baseline cultures, and cultures treated with 1 µM PCS showed no change in cell viability in comparison with baseline cultures.

After having monitored the dose-dependent impact of PCS on osteoclast survival and function, we studied the effects of vitamin D on rat osteoclasts in PCS- and acetazolamide-treated rat osteoclasts, expecting a possible positive influence on the osteoclast survival rate due to this treatment (unpublished). In this experiment we observed a dose-dependent reduction in actin ring numbers due to exposure to PCS, the lowest actin ring number thus being observed with 100 µM PCS, but this decline was not restored by vitamin D treatment (Fig. 5). Control cultures treated with vitamin D contained more actin rings than control cultures incubated in vitamin D-free culture media, but the difference in actin ring numbers was not statistically significant. According to other published results, an increase in osteoclast numbers due to vitamin D would nevertheless be expected (Suda et. al 1995).

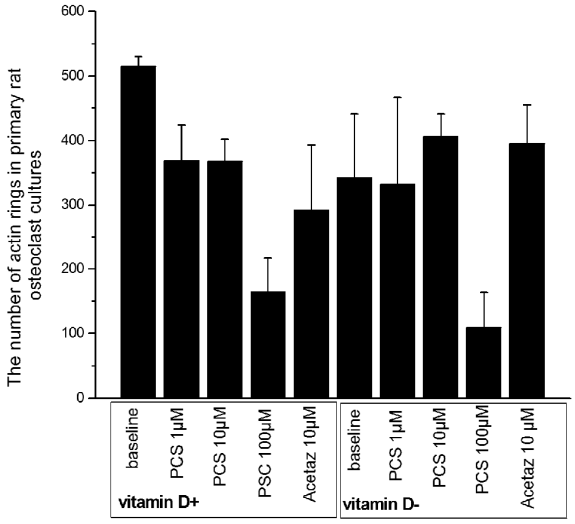


Figure 5: Effects of vitamin D on actin ring numbers in primary rat osteclast cultures in the presence of PCS or acetazolamide.

To examine the effects of membrane-bound carbonic anhydrase inhibition on TRACP expression by rat osteoclasts, we cultured primary rat osteoclasts treated with 1, 10 or 100 µM concentrations of PCS in a hypoxic chamber for 3 days (unpublished). We used both cellular staining and a culture media immunoassay to analyze the amount of TRACP in these cultures. To our surprise, we observed that PCS caused a dose-dependent increase in the amount of TRACP with both detection methods (Fig 6 a and

b). This increase was partially abolished in hypoxia, a feature that could possibly be explained by the generally reduced number of osteoclasts in hypoxic cultures (Fig. 6 b). Interestingly, cultures treated with 10 µM PCS and subjected to hypoxia showed an increased cell survival in contrast to all other groups cultured in hypoxia (Fig. 6 b).

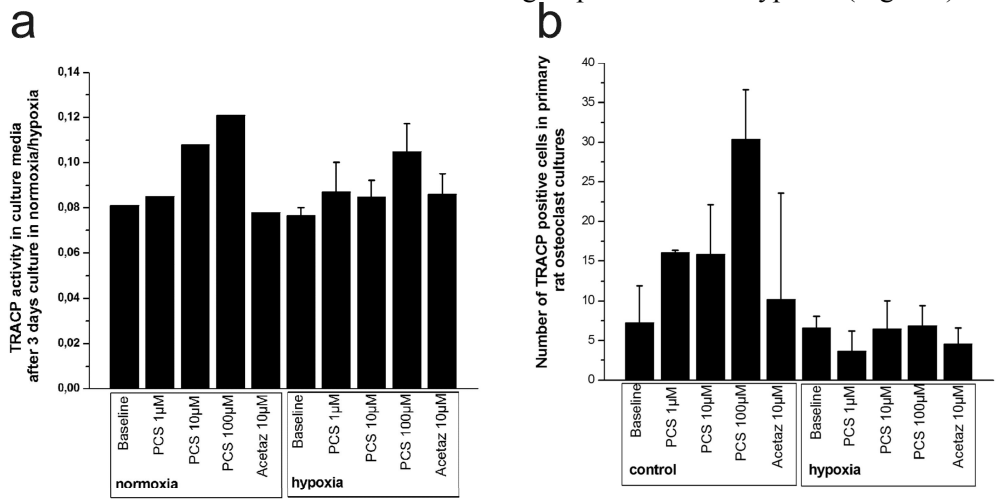


Figure 6: a) TRACP activity in culture media exposed to different concentrations of PCS in hypoxia/normoxia b) Number of TRACP positive cells in primary rat osteoclast cultures exposed to PCS in hypoxia/normoxia.

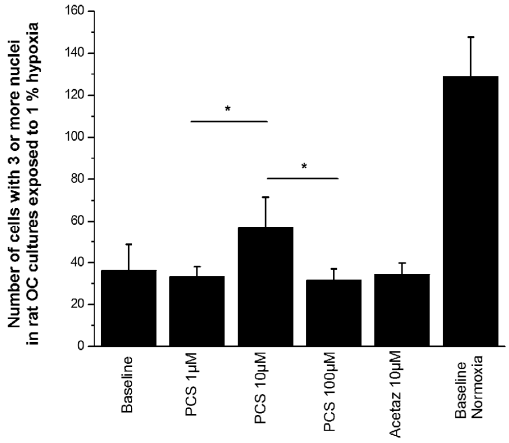


Figure 7: The number of multinuclear osteoclasts in cultures exposed to 1 % pO_2.

5.2 Electroneutral NBCn1 in osteoclasts (II)

5.2.1 Expression of NBCn1 in rat osteoclasts

We studied the expression of the electroneutral sodium-bicarbonate co-transporter NBCn1 in rat osteoclasts in order to clarify the role of bicarbonate transporter proteins in bone resorption. A preceding study had shown that M-CSF causes NBCn1-mediated intracellular alkalosis and promotes cell survival in non-resorbing osteoclast-like cells (Boyer et al. 2007). Thus, we began by studying resorbing rat osteoclasts. We

processed three-day old rat pup bone sections with immunohistochemistry in order to define the subcellular localization of NBCn1 and found that NBCn1 was expressed at protein level in the rat osteoclast ruffled border (II, Fig.1). In addition, we observed NBCn1 immunolabeling in the periosteum, in both proliferative and hypertrophic artilage matrix and in the articular cartilage matrix. We also prepared primary rat osteoclast cultures and monitored NBCn1 mRNA expression after 4, 24 and 48 hours of culturing in the presence or in the absence of RANKL. Interestingly, in the absence of RANKL, the NBCn1 mRNA levels were low after the first 4 hours of culture but increased significantly after 24 hours. In contrast, in the presence of RANKL, the expression of NBCn1 mRNA increased already after 4 hours of culture, decreasing after 24 hours of culture (Figure 8). This simple experiment suggests that osteoclasts produce more NBCn1 during their activation, and the increased in NBC1n expression might be sped up by RANKL, a potent stimulator of bone resorption.

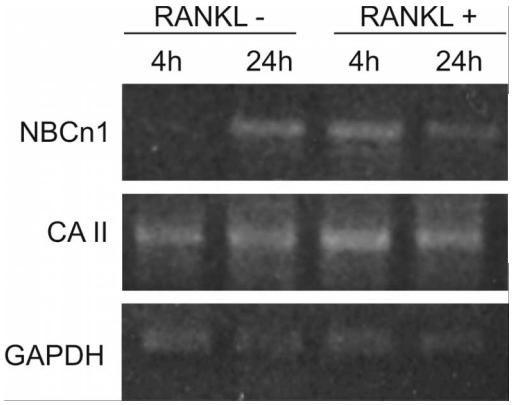


Figure 8: Effects of RANKL on NBCn1 and CA II mRNA expression. GAPDH represents a house-keeping gene.

5.2.2 NBC1n in osteoclastogenesis and osteoblastogenesis

Isolated CD^{14+} cells from human peripheral blood were cultured on glass and bovine cortical bone slices in the presence of RANKL and M-CSF to induce osteoclast formation in order to study NBCn1 expression in these cells. Cells were either fixed or total RNA was extracted at three time points (after isolation, after 3 and 7 days of culture). We discovered the first significant increase in NBCn1 mRNA already after 3-day culture on bone, as compared to undifferentiated CD^{14+} cells (II, Figure 2B). Another strong induction in NBCn1 mRNA expression was detectable after 7 days, as compared to undifferentiated CD^{14+} cells, and at this time point numerous mature multinuclear osteoclasts with bone resorption capacity were observable in the osteoclast cultures. Hence, we confirmed that NBCn1 expression was increased during osteoclastogenesis and osteoclast activation also in humans.

Furthermore, we used confocal microscopy to evaluate the intracellular localization of NBCn1 in human osteoclasts (II, Figure 3). We perceived that immunolabeling for NBCn1 concentrated to the bone-facing areas of resorbing osteoclasts and especially inside the actin ring, where bone resorption occurs. Some staining was detectable also outside the actin ring but at areas where a resorption pit could be seen with phase

contrast microscopy (Figures 3B and C). In addition, some diffuse staining was observed at cytoplasmic areas where Golgi/ER organelles are located.

We also used mesenchymal stem cell cultures to monitor whether osteoblasts would also express NBCn1. We noticed that mRNA levels of NBCn1 remained unchanged during the 8-day culture period and confirmed that NBCn1 is not expressed in human osteoblasts. Hence, when we compared NBCn1 mRNA expression levels between mature human osteoclasts (day 7) and osteoblasts (day 8), a 9.7 ± 0.42 times higher NBCn1 expression was observed in the bone resorbing osteoclasts than in osteoblasts.

5.2.3 Effects of acidosis on NBCn1 expression in rat osteoclasts

We set up an animal model to study the impact of chronic metabolic acidosis on rat osteoclasts. 20- to 30-day-old rats were orally treated with 0.28 M ammonium chloride ($NHCl_4$) for 2 weeks to obtain chronic metabolic acidosis, and the development of acidosis was evaluated by measuring urine pH (II, Table 1). After two weeks the animals were sacrificed, the long bones were dissected and processed using an immunohistochemical protocol. In this experiment, we were able to observe a clear increase in the NBCn1 protein labeling intensity in rat osteoclasts exposed to chronic metabolic acidosis (II, Fig. 2). Both the ruffled borders and the cytosol of osteoclasts showed a clear increase in the NBCn1 immunolabeling intensity.

Furthermore, we explored whether NBCn1 mRNA expression would be sensitive to extracellular pH changes *in vitro*. To study this, primary rat osteoclasts were cultured on bovine bone slices at three different pH levels: pH 7.2 (control), pH 6.6 / pH 6.8 (acidic) or pH 7.6 (alkaline). A part of the cultured cells were fixed and a part of the cultures were used for total RNA extraction after 4 hrs or 24 hrs of culturing. In all three culture groups osteoclast morphology appeared normal and no changes in the osteoclast numbers or viability were detected. NBCn1 mRNA levels were elevated in both acidic and alkaline cultures after 4 hours of culturing as compared to control. An increase in the NBCn1 mRNA expression was also observed after 24 hours of culturing in response to both acidic and alkaline environments as compared to control (Figure 9).

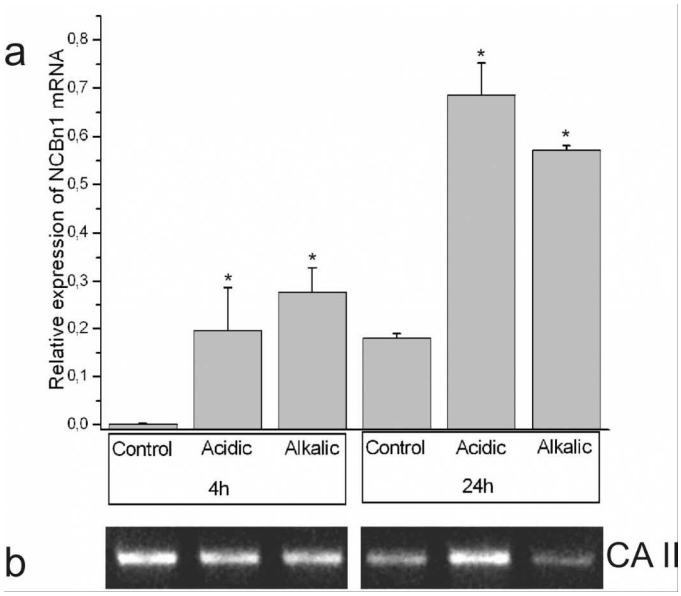


Figure 9. The effects of acidic (pH 6.6-6.8) or alkaline (pH 7.6) culture media on NBCn1 mRNA expression. Control cultures were maintained at pH 7.2. Relative expression was counted by dividing the relative optical densities of NBCn1 by GAPDH (a). RT-PCR was repeated two times from separate samples (n=3 in each sample) and the data is shown as mean +/- SD (ANOVA: $p = 6.9 \times 10^{-5}$). The effects of extracellular pH on CA II expression are shown in section b.

5.2.4 Effects of anoxia on NBCn1 expression in rat osteoclasts

We subjected RANKL-activated primary rat osteoclasts cultured for 1 day to anoxic stress for either 5 or 20 minutes. The aim of the experiment was to assess changes in the NBCn1 mRNA expression in response to severe stress that would immediately interrupt bone resorption and finally lead to osteoclast apoptosis. In this experiment, we used sodium/hydrogen exchanger type 1 (NHE1) primers as a positive control because NHE1 mRNA levels are known to be elevated in response to hypoxic/anoxic stress (Shimoda et al. 2006). With this setting, we were able to see a dramatic decrease in the NBCn1 mRNA levels already after 5 minutes of anoxia compared to control levels (Figure 10). In contrast, the GAPDH and NHE1 mRNA levels remained unchanged and the osteoclast morphology showed no apparent changes even after 20 minutes of anoxic stress. This finding may indicate that apoptotic changes were not yet initiated but the bone resorption process may have been affected, associated with the rapid downregulation of the genes related to osteoclast activation, including NBCn1.

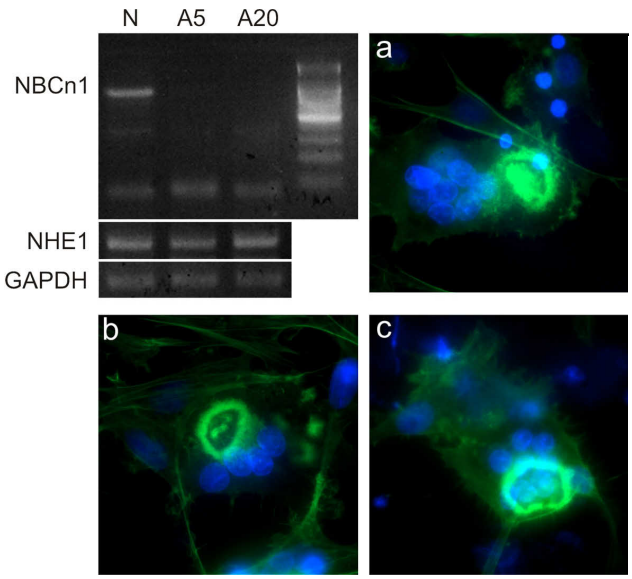


Figure 10. The effects of anoxic stress on osteoclast morphology (a-c) and NBCn1 mRNA expression (d). Cultured osteoclasts were exposed to anoxic stress for 5 (b) or 20 minutes (c) and osteoclasts cultured at normoxia are shown in section a. Apoptosis was evaluated microscopically after DAPI staining (blue nuclei in a-c) and cytoskeletal changes were determined after Phalloidin staining (green in a-c). The bar in a-c corresponds to 20 μm. The mRNA expression levels of NBCn1, NHE1 and GAPDH were evaluated at normoxia (N), or after 5 (marked A5) or 20 minutes (marked A20) of anoxic stress (d).

5.2.5 Downregulation of NBCn1 in osteoclast cultures with shRNAs

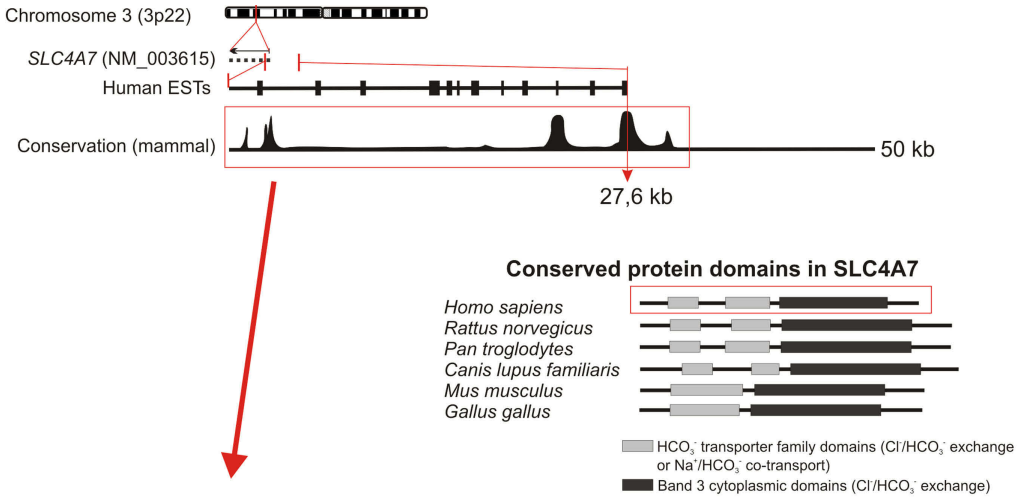
In an attempt to examine the effects of NBCn1 inhibition on primary rat osteoclasts, we designed an experiment including small interfering RNA molecules targeted against NBCn1 during human osteoclastogenesis and osteoblastogenesis. Lentiviral particles containing three *SLC4A7*-specific shRNAs were transduced into CD^{14+} cells and mesenchymal stem cells. CD^{14+} cells were cultured on bone in osteoclastic differentiation media for 6 days before lentiviral transduction, and bone resorption activity was evaluated on day 8 (48h after shRNA transduction, Figure 4A, II). A significant, dose-dependent reduction in the bone resorption capacity and NBCn1 mRNA expression was observed (Figure 4A, II), while the numbers of osteoclasts remained unchanged (Table I, II). Intracellular acidification was increased in shRNA-transduced osteoclasts (Figure 4B, II), as compared to mock-transfected resorbing cells and inactive osteoclasts cultured on cover slips. Lentiviral shRNA transduction showed no significant effects on osteoblast differentiation, cell morphology or viability (Figure 5, II).

5.2.6 Promoter and pathway analysis of the NBCn1 gene sequence

Based on homology with the *SLC4A4* (electrogenic NBCe1), a gene (*SLC4A7*) coding for an electroneutral NBC was cloned from a human skeletal muscle library and called NBC 3. A rat electroneutral NBC was cloned from vascular smooth muscles and called NBCn1, which is also a product of *SLC4A7*. Homology searches showed that the *SLC4A7* gene is well conserved among species (Figure 11), especially humans and rats. While annotation of the rat genome is not complete, it was only possible to perform promoter analysis on human *SLC4A7*. This gene is located in the minus strand of the chromosomal locus 3p22, with multiple ESTs located in the noncoding upstream

region, as shown in Figure 11. Promoter analysis suggested that the actual promoter region seems to be located 27.6 kb upstream of the annotated transcription start site (TSS). CAGE tag analysis revealed that a total of 23 CAGE tag clusters consisting of 225 individual CAGE tags were located at this region while only 1-2 CAGE tag clusters were found near the annotated TSS. Conreal analysis showed that many pH- or hypoxia-regulated transcription factors (TF) were located at the CAGE tag a maximum of 27.6 kb upstream of the annotated TSS.

The interactions between TF binding sites located at 27.6 kb upstream of the annotated TSS were analyzed using the Ingenuity Pathways Analysis (Figure 12). RANKL, which upregulated NBCn1 expression in our osteoclast cultures, is regulated by Early growth response proteins 2 and 3 (EGR2 and EGR3), both TFs that also have binding sites in the *SLC4A7* promoter. RANKL also stimulates the expression genes crucial for bone resorption, namely cathepsin K, TRACP 5b and MMP9.



Cap-Analysis Gene Expression
(CAGE tags for NM_003615)

Bases from transcription start site	Number of cage tage clusters	Total number of cage tags
-4 256	1	1
-1 314	1	3
+1	2	2
+1 911	1	1
+17 491	1	1
+17 706	1	1
+18 133	1	1
+23 120	1	1
+26 333	1	2
+26 546	1	1
+26 873	1	1
+27 242	4	10
+27 376	5	7
+27 478	7	10
+27 522	2	2
+27 585	6	35
+27 630	23	225
+27 736	2	4
+76 743	1	1
+94 490	1	1
+124 827	1	1

Transcription factor binding sites
(Conreal results)

Bases from transcription start site	Length of binding site (bases)	Transcription factor(s)
+27 537	9	E2F-1
+27 608	9	E2F-1
+27 679	12	MAZR
+27 680	7	MAZ
+27 682	13	Egr-1
-"-	13	Egr-2
-"-	13	Egr-3
-"-	13	Egr-4
+27 688	7	MAZ
+27 734	10	AhR
+27 737	7	MAZ
+27 762	15	AhR
-"-	15	Arnt
-"-	10	AhR
+27 765	7	MAZ
+27 767	10	E12
+27 768	9	Tal-1
-"-	9	TAL-1α
+27 769	7	USF
-"-	7	USF-1
-"-	7	USF-1
+27 771	7	MAZ
+27 848	7	MAZ

Figure 11: Promoter analysis of human *SLC4A7* gene encoding NBCn1. On the basis of high interspecies homology and inadequate annotation of rat NBCn1 gene, the corresponding human *SLC4A7* gene (NM_003615) was studied. The noncoding upstream region contained multiple ESTs and conserved areas. A total of 150kb upstream of transcription start site was analyzed for CAGE tags, and the CAGE tag maximum at 27.6 kb was further analyzed for transcription factor binding sites using Conreal. Abbreviations: MAZ, myc-associated zinc finger; MITF, microphthalmia-associated transcription factor; AHR, aryl hydrocarbon receptor; E2F1, E2F-transcription factor 1; ARNT (HIF-1β), aryl hydrocarbon receptor nuclear translocator; EGR, early growth response; ACP5, tartrate-resistant acid phosphatase 5b; CTSK, cathepsin K; MMP9, matrix metalloproteinase 9; TNFSF11 (RANKL), receptor activator of NFκB ligand.

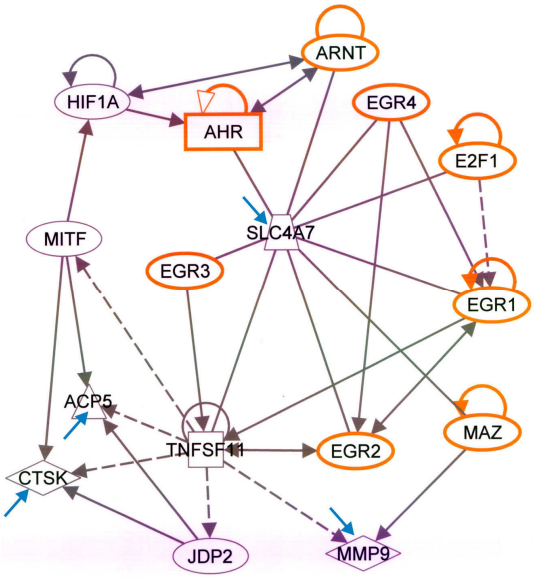


Figure 12: Transcription factors with binding sites at the *SLC4A7* promoter were uploaded to Ingenuity Pathways Analysis for interaction network analysis. The uploaded genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base, and the network was algorithmically generated based on gene connectivity. Transcription factors with binding sites at the *SLC4A7* promoter are shown as orange nodes, and gene products crucial for bone resorption are marked with blue arrows. Nodes are displayed using various shapes that represent the functional class of the gene product. Abbreviations: JDP2, jun dimerization protein.

5.3 Carbonic anhydrase III in cartilage (III)

5.3.1 Expression of CA III in embryonal mouse cartilage

We performed immunohistochemistry on both embryonal and newborn mouse limb sections in order to study CA III expression in developing cartilage. We observed that already on day e14.5, distinct CA III staining was visible in the lacunae of prehypertrophic chondrocytes of our samples, but proliferative cartilage remained negative for CA III (III, Fig. 2 B). A similar CA III staining pattern was observed also on day e16.5 at the interface between hypertrophic cartilage and bone, but its intensity was stronger than on day e14.5 (data not shown). On day e18.5, intense CA III staining was detectable in hypertrophic chondrocytes (III, Fig. 2 B). In addition, newborn mouse limb sections expressed CA III in their hypertrophic cartilage but, in contrast, 20-day-old mice limbs presented only faint CA III positivity in this area. Finally, 2-month-old mouse cartilage showed CA III staining only in some chondrocyte lacunae at the articular surfaces (data not shown) and in a few hypertrophic cells (III, Fig. 2 B). Skeletal muscle that was used as a positive control stained positive, as expected. Quantitative PCR confirmed the high expression of CA III also at mRNA level in embryonal cartilage (III, Fig. 2 A). In metacarpal cultures, intense CA III staining was observed in hypertrophic chondrocytes (III, 1 D, E and I), and the staining was first observed in prehypertrophic chondrocytes located at the centre if the cartilage anlage (III, Fig 1 H).

5.3.2 Co-localization of CA III with ROS in mouse cartilage

We wanted to explore the localization of reactive oxygen species in embryonal mouse limb cartilage. Therefore, we processed snap-freezed mouse limb samples into cryosections and exposed them to carboxy-H2DCFDA, a reagent which emits fluorescent light when oxidized. In this experiment, we observed that cartilage samples

from both days e18.5 and e20.5 presented reactive oxygen species localized at the hypertrophic cartilage and at the proliferative cartilage of the growth plate (III, Fig. 5) as well as at the articular surface of joints (III, Fig. 5). Most ROS on day e18.5 were observable in the hypertrophic cartilage (III, Fig. 5) while, in contrast, most of ROS on day e20.5 were localized in the proliferative cartilage (data not shown). Another interesting finding was that when we compared samples from days e18.5 and e20.5, there was a significant decrease in the fluorescence of carboxy-H2DCFDA in the articular surfaces on day e20.5 (data not shown). In conclusion, possible colocalization of ROS and CA III was observed in our embryological limb cartilage samples.

5.3.3 The effects of oxidative challenge on CA III expression

To evaluate the physiological responses of CA III to altered O2 content, we cultured fetal mouse metacarpi in hypoxia (2% O_2), normoxia and hyperoxia (30% O_2). Genetic responses to this treatment were studied by qPCR to confirm how VEGF and HIF-1 levels changed in the cartilage anlage. As expected, significant increases in VEGFα, type I collagen and HIF-1β mRNA expressions were observed in hypoxia, along with decreased HIF-1α and I type X collagen mRNA expressions. The morphological analysis of cultured metacarpals showed increased calcification but only limited hypertrophy in hypoxia, as compared to normoxia. The development of the primary marrow cavity was abolished in hypoxia (III, Fig. 3 A), although an increased cell density was observed at these areas, resembling proliferative chondrocytes. In hyperoxia, increased expression of type X collagen was observed while all hypoxia-responsive genes were downregulated. An increase in the width of the hypertrophic cartilage zone was observed in hyperoxia, but the development of the primary marrow cavity was similar to the normoxic samples (III, Fig. 3 A). It was of interest to note that the expression of CA III mRNA was substantially down-regulated in hypoxia, but it was also decreased in hyperoxia, as compared to normoxia (III, Fig. 4 A). When the protein expression was analyzed by immunohistochemistry, a significant increase in CA III protein was observed in hypoxia, localized mostly in the extracellular matrix at areas underlining the developing perichondrium (III, Fig. 4 B). Also in hyperoxia, an increase in CA III protein staining was observed at the hypertrophic cartilage matrix, as compared to normoxia. These data clearly show how physiological responses on the mRNA and protein levels may be totally opposite.

6 DISCUSSION

6.1 Bicarbonate transport in osteoclasts

6.1.1 CA IV, CA XIV and NBCn1 participate in bone resorption

Systemic pH can be defined as the ratio of the amount of acids, such as lactate and carbonic acid, to the amount of bases in systemic fluids in humans. The most important base that buffers acids in the body is bicarbonate, the availability of which is controlled by multiple mechanisms: absorption of dietary bases in the gastrointestinal tract, absorption/reabsorption of bicarbonate by the kidneys and binding/release of bicarbonate from bone. It is clear that the bicarbonate supply is secured by many overlapping mechanisms because it is constantly needed in physiological activities in the body and because its depletion could result in metabolic acidosis, a life-threatening condition for the whole system.

We have described mechanisms of bicarbonate transport in osteoclasts. First, we determined the subcellular localization of three bicarbonate transport proteins CA XIV, CA IV and NBCn1 in rat and human osteoclasts. Furthermore, we explained that these proteins play prominent roles in osteoclastic bone resorption, and that inhibition of these proteins with shRNAs or selective inhibitors results in abnormal osteoclast morphology and reduced bone resorption capacity. Additionally, we have come to the conclusion that a bicarbonate transport metabolon may be present in osteoclasts, and presume that this metabolon is responsible for the disposal of excess bicarbonate and carbonate from the resorption lacuna during bone resorption.

In article I, we found that inhibition of carbonic anhydrase activity on the non-bone facing plasma membrane, using a cell membrane-impermeable carbonic anhydrase inhibitor PCS, resulted in severe defects in osteoclast morphology, lack of attachment to bone and inhibition of acid release to the resorption lacuna. Furthermore, cell survival was diminished in our cultures when using 100 μM PCS. This observation can either be caused by 1) direct toxic effects of PCS, independent of carbonic anhydrase inhibition leading to necrosis, or 2) membrane-bound carbonic anhydrase inhibition by PCS, leading to changes in cellular signaling and eventually to programmed cell death. It is rather difficult to know which one of the options we encountered in our study. Nevertheless, we observed that acetazolamide, a sulfonamide inhibitor with a soluble structure but otherwise similar to PCS, was not able to induce cell death or have an impact on actin ring numbers when administered to primary rat osteoclast cultures. Hence, we conclude that a direct toxic effect of PCS on osteoclasts is at least not the only mechanism that affected osteoclasts in our cultures but could, nevertheless explain reduced cell survival due to higher concentrations of PCS. This uncertainty can, however, be seen as a limitation of our study.

An intriguing observation was also made in article I about primary rat osteoclasts exposed to 10 μM PCS: increased cell survival was detected in these cultures in

comparison with baseline, and the difference remained constant even after exposure of osteoclasts to severe hypoxia, a strong pro-apoptotic stimulus. Since it can be assumed that the impact of PCS on osteoclasts is associated with inhibition of extracellular carbonic anhydrase activity, it is probable that this effect also explains the enhanced cell survival. Inhibition of carbonic anhydrase activity on a resorbing osteoclast cell membrane directly leads to accumulation of bicarbonate in the immediate vicinity of the basolateral osteoclast cell membrane. This shift toward extracellular alkalosis might explain our observation concerning increased cell survival, because systemic alkalosis has previously been shown to inactivate osteoclasts and to possibly reduce osteoclast apoptosis (Arnett, 2008). The mechanism could, in fact, involve the action of NBCn1 that has been shown to contribute to enhanced CSF-induced survival of osteoclast-like cells. Nevertheless, more studies are warranted to elucidate this option.

In article II, we reported that an electroneutral sodium-dependent bicarbonate co-transporter NBCn1 is expressed at the ruffled border of resorbing rat osteoclasts. We found that NBCn1 mRNA expression is readily induced by factors related to osteoclast activation, such as acidosis and exposure to RANKL. To our surprise, we found that extracellular alkalosis too induced NBCn1 mRNA expression in primary rat osteoclast cultures *in vitro*. This finding is not consistent with previous studies in this field. Nonetheless, it can be speculated that the increase in NBCn1 mRNA might possibly be linked to the bi-directional function of NBCn1, since NBCn1 has been reported to be able to transport bicarbonate both ways (into and out of the cell) when sodium is available. This dual function could be necessary for the protection of osteoclasts from an excessively alkaline environment. Furthermore, induction of alkalosis by sodium hydroxide provides osteoclasts with a sodium load that could possibly also have an effect on the expression of NBCn1. Nonetheless, we did not perform any experiments to further elucidate this unexpected result, and it can be seen as a limitation in our study.

6.1.2 Bicarbonate transport - the long-ignored necessity in osteoclasts

Osteoclasts are unique giant cells with only one known main function: bone resorption. Many visionary studies conducted as early as the 1980s and 1990s thoroughly investigated cellular machinery responsible for the implementation of this function. Multiple experiments have confirmed the role of proteins that enable formation of the ruffled border membrane domain in osteoclasts and efficient transportation of protons and chloride. Nevertheless, the flip-side of the coin has gained little attention and has, in my view, been severely underestimated. Transport of bicarbonate, a necessary counterforce for the transport of acidic substances, has been forgotten in scientific discussion.

We have shown in this study that bicarbonate transport proteins are of significance in rat and human osteoclasts *in vitro*. Our investigations have shown that deletion of these proteins from functional osteoclasts has effects that may even terminate bone resorption. For the first time, we were able to conclude that bicarbonate transport proteins are biologically necessary in functional osteoclasts -but this was no surprise to us! On the contrary, it is very logical that these proteins are needed in osteoclasts,

bearing in mind basic chemistry: for every proton, an alkaline ion arises from the original compound. Furthermore, the skeleton is an obvious site for storing many substances, including carbonate and bicarbonate. It is reasonable from a systemic point of view to ensure the liberation of storage material at any time it may be required, which is clearly the job of osteoclasts. In this way, rapid changes in the resorption activity of osteoclasts ensure availability of alkaline ions for systemic needs.

Another important hypothesis that we resolved in this part of the study was that osteoclasts possess appropriate machinery for a bicarbonate transport metabolon. Although the existence and especially the properties of bicarbonate transport metabolons have been under debate through the last decade, recent studies have confirmed the existence of these metabolons in many cell types and multiple associated proteins such as CA II, CA IV, CA XIV, NHE proteins and AE proteins as well as sodium/bicarbonate transport proteins (see for example Casey et al. 2009; Gonzalez-Begne et al. 2007). Results indicate that in most cases, direct physical binding among the bicarbonate transport metabolon proteins may be detected. Furthermore, *CA XIV* knockout mice at least have already been shown to highly resemble *AE3*-deficient mice, which further suggests that bicarbonate transport metabolon proteins occupy a common important function and act in a chain in order to move bicarbonate efficiently. In osteoclasts, the existence of bicarbonate transport metabolons has not been suggested before and therefore our results may open up new perspectives for future experimentation in this field. For instance, co-immunoprecipitation used to assess the possible physical binding of proteins of the bicarbonate metabolon in osteoclasts could give further information on this issue.

Both bone resorption and the function of intracellular CA II result in an excess of intracellular bicarbonate, which cannot remain inside the actively resorbing osteoclast. In our model of osteoclastic bicarbonate transport metabolon, bicarbonate in the bone resorption lacuna is carried into the osteoclasts by NBCn1 located on the ruffled border. Thereafter, intracellular bicarbonate is discarded from osteoclasts by AE(2) proteins located on the basolateral membrane. Then CA XIV, the active CA domain of which is located on the outside of osteoclast cell membrane, ensures the conversion of bicarbonate to pH-neutral agents (water and carbon dioxide). In this way, osteoclasts escape the formation of extracellular alkalosis by bicarbonate in their vicinity. Hence, we suggest a functional metabolon for bicarbonate transport in osteoclasts, the components of which include CA II, NBCn1, AE proteins and CA XIV (see Figure 13).

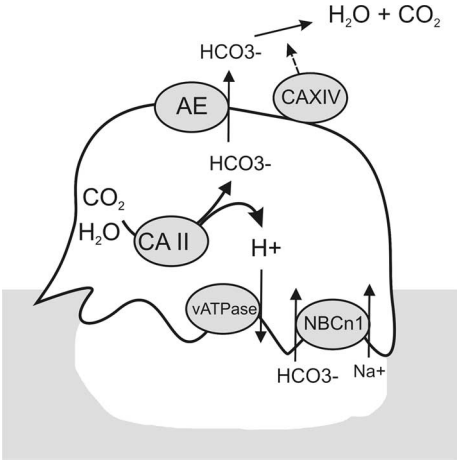


Figure 13: Our suggestion for a rat osteoclast bicarbonate transport metabolon. CA II, carbonic anhydrase II; vATPase, vacuolar ATPase, NBCn1, sodium-dependent bicarbonate co-transporter; AE, anion exchanger protein; and CA XIV, carbonic anhydrase XIV.

6.2 Oxidative metabolism in cartilage

6.2.1 Hypoxia and ROS - important mediators for cartilage development

The development, proliferation and vitality of chondrocytes have been shown to have a direct link to hypoxia, an important regulator for this tissue. Nevertheless, recent observations also indicate that reactive oxygen species (ROS) arising in cells due to altered oxygen content could mediate at least some of the effects of hypoxia on chondrocytes. This has been argued by referring to studies indicating that the existence of ROS could be a physiological finding in cartilage exposed to normal or high oxygen levels, and that reducing amounts of ROS due to severe hypoxia could hence represent a pathway for modulating chondrocyte function (Milner and Wilkins, 2007).

We studied the expression of CA III in embryonal mouse cartilage in order to clarify the role of oxidative metabolism in these cells. Carbonic anhydrase III has previously been shown to play a role as a mechanism with which muscle cells defend themselves against protein oxidation and increased ROS formation. Moreover, previous studies have shown that NIH cells overexpressing CA III are able to maintain a lower state of intracellular ROS and to escape apoptosis (Räisänen et al. 1999). Thus, CA III function has previously been linked to ROS scavenging.

We found in our experiments that carbonic anhydrase III is expressed in mouse cartilage from the early days of embryonal development until mature adulthood. Interestingly, strongest expression of CA III both at protein and mRNA level was observable during embryonal cartilage development when compared to samples obtained after mouse birth. Moreover, the localization of CA III was different in embryonal samples compared with postpartum samples, since most of CA III protein in embryonal cartilage localized in the hypertrophic cartilage which, in turn, totally lacked CA III after birth. It is not clear where this differential expression of CA III in cartilage derives from, but it could be related to changing oxidative conditions in the vicinity of proliferating and maturing chondrocytes during limb growth and have a link to ROS production. In support of this we showed that embryonal cartilage samples

contain reactive oxygen species and that these ROS are mostly detectable at the same sites with CA III expression. Thus, if the function of CA III in cartilage and in muscle is alike, CA III expression in hypertrophic chondrocytes could be needed for preventing or for at least slowing down irreparable protein oxidation due to ROS formation in chondrocytes.

We can only speculate as to why the amount of CA III immunoreactivity decreases immediately after the birth of the animals. We propose that this phenomenon might reflect alterations in the oxygen levels and in the pace of ROS formation during limb cartilage growth. During the early phases of embryonal limb chondrogenesis the size of avascular cartilage is small, and the level of hypoxia in hypertrophic chondrocytes may be advantageous, allowing proper chondrocyte maturation and carrying a low risk of protein oxidation and apoptosis. While the areas of proliferative and hypertrophic cartilage develop and grow higher, however, hypertrophic chondrocytes might fall into such low oxygen levels that the risk of cellular damage is high and that their metabolism is downregulated. In order to stay alive long enough, hypertrophic chondrocytes are therefore forced to express HIF-1 and VEGF which have been observed to promote chondrocyte survival. VEGF expression is first detected in mouse hypertrophic cartilage at e14.5-15.5 as it invites blood vessel sprouts to the area (Carlevaro et al. 2000; Zelzer et al. 2002). At the same time point, the expression of CA III in the hypertrophic cartilage increases according to our study, providing hypertrophic chondrocytes with protection from protein oxidation, and allowing the enlargement of cartilage in spite of an obvious proapoptotic stimulus by the very hypoxic environment. The earliest blood vessel sprouts have been found to have invaded the mouse limb hypertrophic cartilage at e15.5 for the formation of the primary ossification centers (for review, see Riddle *et al.* 2009), simultaneously providing a load of oxygen to the area. At this time, a need to protect chondrocytes from oxidative damage is obvious, since a sudden supply of oxygen at the ossification centers exposes nearby cells to reoxygenation reactions and a significant flux of ROS. CA III could possibly play an important role in this regard, inhibiting the early death of chondrocytes and thus allowing formation of a wide enough hypertrophic zone and finally, the formation of the cartilage-bone interface. Later, after the birth of the mouse, the width of the growth plate has been stabilized and the blood circulation in the area is sufficient to support bone formation, and CA III expression may reduce to allow timely hypertrophic chondrocyte death and formation of bone nodules at the cartilage-bone interface.

It is important to realize that the fall of hypertrophic chondrocytes into a deep hypoxic state and exposure to ROS after blood vessel sprouting may be prerequisites for the development of the future cartilage-bone interface and not just “side-effects” of fast limb development. This argument is linked to the pivotal role of VEGF in endochondral bone formation. First of all, hypoxia is an important stimulator for HIF and VEGF expression. Secondly, VEGF has been observed to regulate osteoblast function and to induce bone formation by osteoblasts. Thirdly, blood vessels sprouting due to expression of VEGF is needed for transferring both osteoblast and osteoclast precursor cells to hypertrophic cartilage areas for initiation of bone formation. This

notion is supported by multiple studies showing that the disruption of blood vessel invasion of hypertrophic cartilage and inhibition of VEGF expression both lead to decelerated replacement of chondrocytes by bone and an expanded hypertrophic zone (Trueta and Buhr 1963, Zelzer *et al.* 2002), meaning that the timely appearance of blood vessel sprouting in hypertrophic cartilage is essential as regards bone formation. Thus, it is clear that VEGF provides not only pro-angiogenic stimulus to hypertrophic cartilage, but it also promotes the formation of the cartilage-bone interface, and CA III may be a supporting factor in this process.

7 SUMMARY

Our aims in this study were to clarify regulation mechanisms for acid-base balance and oxidative metabolism in calcified tissues. We concentrated especially on exploring bicarbonate transport in osteoclasts and the role of various carbonic anhydrases in osteoclasts and chondrocytes. These issues have not been studied troughly before, albeit understanding such basic cellular functions may provide valuable tools for later pharmaceutical development.

Our findings indicate that bicarbonate transport is an essential part of bone resorption, and our data shows a clear decrease in the bone resorption capacity of osteoclasts when bicarbonate transport proteins are inhibited. It has been known for long that both carbonate and bicarbonate are released from the bone matrix in great amounts during bone resorption. Neverthless, our study is the first one to link both membrane-bound carbonic anhydrases and an electroneutral sodium-dependent bicarbonate transporter NBCn1 to actively resorbing osteoclasts. Furthermore, our study has refered to the existence of a bicarbonate transport metabolon in osteoclasts. Therefore, we have provided new insights to the bone resorption process and suggested a pivotal role for bicarbonate transport proteins in bone mineral metabolism.

We also defined the expression of a hypoxia-related protein, carbonic anhydrase III in embryonal mouse cartilage in the second part of our study. Hypoxia, an important regulator for chondrocyte function, is linked to the production of reactive oxygen species which can be harmful to cells. We are the first to provide information on CA III which may protect hypertrophic chondrocytes from oxidative damage and apoptosis when they are exposed to oxidative challenges such as reactive oxygen species during embryonal skeletogenesis. Our data increases knowledge in the formation of the cartilage-bone-interface, a premise to trabecular bone formation and proper limb development. These experiments may also in the future lead to the resolving of the physiological function(s) of CA III, an area that has remained unsolved for decades.

In conclusion, we have studied in detail two aspects of calcified tissue metabolism. Our findings provide new insights to the complexity of cellular functions in these tissues and might help scientists to overcome therapeutical challenges related to diseases of bone and cartilage. More specifically, these results suggest NBCn1, CA IV, CA XIV and CA III as novel targets for drug development.

8 ACKNOWLEDGEMENTS

This study was carried out at the Department of Cell Biology and Anatomy, Institute of Biomedicine, University of Turku during years 2004-2010. A part of the study was conducted in the Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Taegu, Korea. I want to thank the head of the Institute of Biomedicine, Professor Pirkko Härkönen, the head of the Department of Cell Biology and Anatomy, Professor Juha Peltonen, and former heads Professor Kalervo Väänänen and Professor Emeritus Risto Santti for providing excellent facilities and for our convenient working environment at the University of Turku. Thanks go to Professor Tae-Hwan Kwon for providing me with the wonderful opportunity and great facilities to carry out my projects in Korea during spring 2007. I also want to thank the Director of the Turku Graduate School of Biomedical Sciences (TUBS), Professor Olli Lassila, for support and advice during this project.

My warmest gratitude belongs to my supervisor Docent Tiina Laitala-Leinonen, whose devotion to research has been a continuous source of inspiration for me during this thesis project and medical studies. Tiina´s motivation, expertise, multiple talents, friendliness and intelligence are amazing! Our discussions and brainstorming sessions have really opened my eyes to research. Your endless support has guided me through these studies, and I know that your advice will stay in my heart during my future career. I cannot thank you enough.

I also want to express my sincerest gratitude to Professor Tae-Hwan Kwon, who let me work in his research group during spring 2007 in Taegu, Korea. I felt very welcome in Korea from day one because of your friendliness and continuous support. Working at the Kyungpook National University was an unforgettable experience for me, and I enjoyed the enthusiastic atmosphere in your laboratory very much. Your kindness, intelligence and insight in the field of biomedical research have been a real motivation to me. I want to thank you for your help and I hope that our friendship continues in the future. I would also like to take the opportunity to thank your students and laboratory personnel who were extremely well-motivated and skillful and with whom I made many friends.

My thesis supervisory committee is acknowledged for providing advice and suggestions during my studies. Professor Kalervo Väänänen has been a major source of support during this study. His unique experience and insight in bone biology have been essential for this study. His belief in my projects has carried me through some challenging times. Thanks go to Professor Seppo Parkkila for providing expertise in our carbonic anhydrase studies. His collaboration during the first steps of this project was invaluable.

I want to acknowledge my coauthors, whose contribution to this project has been of major importance. Thanks go to Professor Claudiu Supuran at the University of

Florence, Italy, for his excellent knowledge in carbonic anhydrase inhibition. Thanks go to Dr. Anna-Marja Säämänen for interesting discussions during our CA III study and for opening my eyes to cartilage research. Thanks go to fellow PhD student Jussi Heinonen for his collaboration during our CA III studies, and for friendship. Thanks go to PhD student Anne Seppänen for introducing me to computer analysis, for conducting the promoter analysis in this study and for her friendship. Thanks go to Professor Silvia Pastorekova for providing the CA IX antibody and to Professor Søren Nielsen for providing the NBCn1 antibody.

Warm thanks go to all former and present members of Boneheads, since you have provided so much friendship during these years! I want to dedicate my special thanks to Salla Laine, Mirkka Hirvonen, Katja Fagerlund, Jonas Nyman, Johanna Likojärvi, Kaisa Ivaska, Hannele Ylipahkala, Terhi Heino, Sari Alatalo, Yi Sun, Guoliang Gu, and ZhiQi Peng for sharing the ups and downs during this project. Warm thanks also go to Salla Laine, Niina Loponen and Vivian Visnapuu for sharing the office and coffee breaks with me for a few years, and to Jonas for interesting discussions during my studies. Thanks go to Professor Teuvo Hentunen for many pieces of advice and for telling the best jokes in the department! Thanks go to Dr. Jukka Vääräniemi for his endless patience when guiding me in the use of microscopes.

I also want to express my gratitude to the entire staff of the Department of Cell Biology and Anatomy. Special thanks go to Iris Dunder and Soili Huhta, whose friendly attitude and help in various matters have been essential on many occasions. Moreover, I want to thank the whole lab personnel for their excellent technical assistance. Thanks also go to Nanna Merikoski, Paula Pennanen, Anneli Kurkela and Paula Pernilä for our fun moments in the lab. Thanks go to Sarah Hollis and Hilkka Salmén for proofreading the thesis.

Warm thanks and love go to my friends, with whom I have shared the ups and downs of this project. Special thanks go to the "Spice Girls+R": Kaisa, Tiina, Venla, Reetta, Elisa and Riku: you are the greatest friends! I also want to thank Essi, Mirva, Kirsi, Aino, Jonna and the "Iidesjärven lenkkiporukka" for sharing your opinions, thoughts and friendship with me.

I can never express enough gratitude and love to my family: my parents Markku and Heljä and my sisters Miia and Maria, my parents-in-law Leena and Simo and my brother-in-law Timo. I would not have made it this far without you. Thanks and love to my daughter Aura for keeping mummy´s feet on the ground. You are my precious little sunshine!

Finally, thanks go to my husband Tuomo for his unlimited love and support during our years together. You have always encouraged me to make my dreams come true. I love you!

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