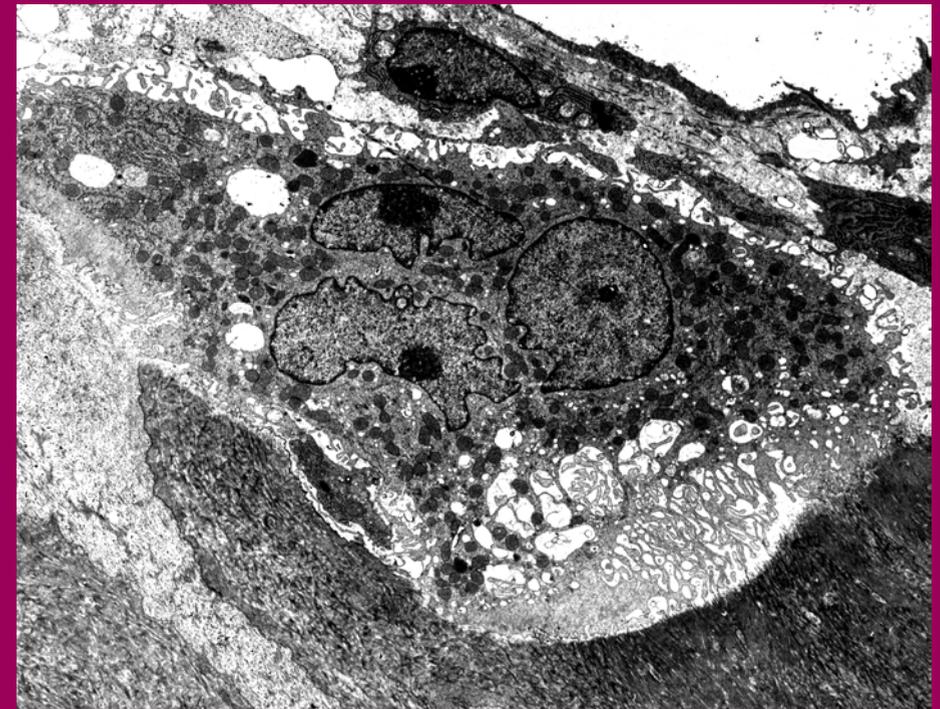


Thesis for doctoral degree (Ph.D.)  
2007

# Studies on the phenotype and function of osteoclasts using osteopetrotic and rachitic animal models



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Karin Hollberg



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Institutet**

Stockholm 2007

“All that we are is the result of what we have thought. The mind is everything.  
What we think, we become.”

- Buddha

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To Isabel, Jesper  
& Erik

# ABSTRACT

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Osteoclasts are multinucleated bone-resorbing cells that have been implicated in a variety of skeletal diseases e.g. osteoporosis, rheumatoid arthritis and skeletal metastasis. Under physiological conditions, the osteoclast participates in ossification during longitudinal bone growth as well as in bone remodelling during adulthood. Bone resorption is initiated by attachment of osteoclasts to the surface of bone to be resorbed by interaction with mineral-associated adhesive proteins. This interaction also drives formation of specialized membrane domains called ruffled borders and sealing zones leading to cell polarization necessary for enclosement of the degradative process in a discrete extracellular area. In this resorption lacuna, local acidification leads to dissolution of mineral, and secretion of proteolytic enzymes e.g. cathepsin K (Ctsk) and matrix metalloproteinase (MMP)-9 completes degradation of the exposed collagen. Another secreted osteoclast enzyme, tartrate-resistant acid phosphatase (TRAP), is a protein phosphatase with suggested roles in regulating osteoclast adhesion, migration and matrix degradation.

In this thesis, animal models with the skeletal diseases rickets and osteopetrosis were employed to study the relation between ultrastructural morphological characteristics of osteoclasts and secretion of biochemical resorption markers e.g. Ctsk, MMP-9 and TRAP. Using two different models of rickets e.g. induced by dietary restriction of phosphate and vitamin D in young rats and transgenic mice overexpressing the phosphate-regulating FGF-23 in osteoblasts, it was demonstrated that mineralization of bone matrix is required for osteoclast polarization but not for secretion of proteolytic enzymes and that matrix mineralization is protective for and limits collagen degradation. It is concluded that the validity of osteoclast polarization as an ultrastructural morphological indicator of osteoclast resorptive activity is restricted to bone with normal mineralization. Moreover, genes encoding mineralization-promoting proteins are activated in osteoblasts from mice with hypophosphatemic rickets, indicative of a compensatory mechanism to favour mineralization.

TRAP is synthesized as an inactive monomeric precursor requiring proteolytic processing to become an active enzyme. Proteolytic processing of TRAP was altered in a subpopulation of metaphyseal osteoclasts in a mouse strain where the Ctsk gene was inactivated, implicating Ctsk as a regulator of TRAP processing in vivo and supporting the concept of functional heterogeneity of osteoclasts depending on their precise anatomical localization within the skeleton. Moreover, the intracellular distribution and secretion of monomeric and proteolytically processed TRAP was altered in Ctsk-deficient osteoclasts, providing evidence for a novel role for Ctsk in the regulation of intracellular membrane traffic in osteoclasts. The possibility that TRAP is the common denominator for this vesicular transport regulation was indicated by the observation of an accumulation of cytoplasmic vesicles in osteoclasts from mice with an inactivated TRAP gene. In addition, whereas Ctsk was secreted by both polarized and non-polarized osteoclasts, TRAP was secreted only by polarized osteoclasts under certain conditions, suggesting different regulation for secretion of these enzymes as well as an action of TRAP later than Ctsk in the matrix degradative phase of the resorption sequence.

# LIST OF PUBLICATIONS

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- I. Karin Hollberg<sup>1</sup>, Joakim Nordahl<sup>1</sup>, Kjell Hultenby, Silwa Mengarelli-Widholm, Göran Andersson, Finn P Reinholt  
Polarization and secretion of cathepsin K precede tartrate-resistant acid phosphate secretion to the ruffled border area during the activation of matrix-resorbing clasts.  
Journal of Bone and Mineral Metabolism 23: 441-9, 2005.
- II. Karin Hollberg, Richard Marsell, Maria Norgård, Tobias Larsson, Kenneth B Jonsson, Göran Andersson  
Osteoclasts in rachitic FGF-23 transgenic mice efficiently degrade bone matrix.  
Submitted manuscript
- III. Karin Hollberg, Kjell Hultenby, Alison Hayman, Timothy Cox, Göran Andersson  
Osteoclasts from mice deficient in tartrate-resistant acid phosphatase have altered ruffled borders and disturbed intracellular vesicular transport.  
Experimental Cell Research 279(2): 227-38, 2002
- IV. Serhan Zenger<sup>1</sup>, Karin Hollberg<sup>1</sup>, Jenny Ljusberg, Maria Norgård, Barbro Ek-Rylander, Riku Kiviranta, Göran Andersson  
Proteolytic processing and polarized secretion of tartrate-resistant acid phosphatase is altered in a subpopulation of metaphyseal osteoclasts in cathepsin K-deficient mice  
Bone, in press, 2007

<sup>1</sup>Shared first authorship

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# ABBREVIATIONS

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|                                      |                                                        |
|--------------------------------------|--------------------------------------------------------|
| 1,25(OH) <sub>2</sub> D <sub>3</sub> | 1,25-dihydroxyvitamin D <sub>3</sub> , calcitriol      |
| Acp5                                 | Acid phosphatase-type 5, TRAP                          |
| ALP                                  | Alkaline phosphatase                                   |
| ATP                                  | Adenosine triphosphate                                 |
| BSP                                  | Bone sialoprotein                                      |
| Cbfa1                                | Core binding factor $\alpha$ 1                         |
| ClC-7 / ClCN7                        | Chloride channel 7                                     |
| CP                                   | Cysteine proteases                                     |
| Ctsk                                 | Cathepsin K                                            |
| CTX                                  | C-terminal cross-linked telopeptide of type I collagen |
| DMP1                                 | Dentin matrix protein-1                                |
| ECM                                  | Extracellular matrix                                   |
| FGF-23                               | Fibroblast growth factor 23                            |
| ICTP                                 | Carboxyterminal telopeptide of type I collagen         |
| IGF-1                                | Insulin-like growth factor-1                           |
| IL                                   | Interleukin                                            |
| M-CSF                                | Macrophage colony stimulating factor                   |
| MEPE                                 | Matrix extracellular phosphoglycoprotein               |
| MMP                                  | Matrix metalloproteinases                              |
| OPG                                  | Osteoprotegerin                                        |
| OPN                                  | Osteopontin                                            |
| PTH                                  | Parathyroid hormone                                    |
| RANK                                 | Receptor of nuclear factor- $\kappa$ B                 |
| RANKL                                | Receptor of nuclear factor- $\kappa$ B ligand          |
| RGD                                  | Arg-Gly-Asn sequence                                   |
| ROS                                  | Reactive oxygen species                                |
| TGF- $\beta$                         | Transforming growth factor- $\beta$                    |
| TNF                                  | Tumor necrosis factor                                  |
| TRAF                                 | TNF receptor-associated factors                        |
| TRAP                                 | Tartrate resistant acid phosphatase                    |
| V-ATPase                             | Vacuolar-type H <sup>+</sup> -ATPase                   |
| VDR                                  | Vitamin D receptor                                     |

# INTRODUCTION

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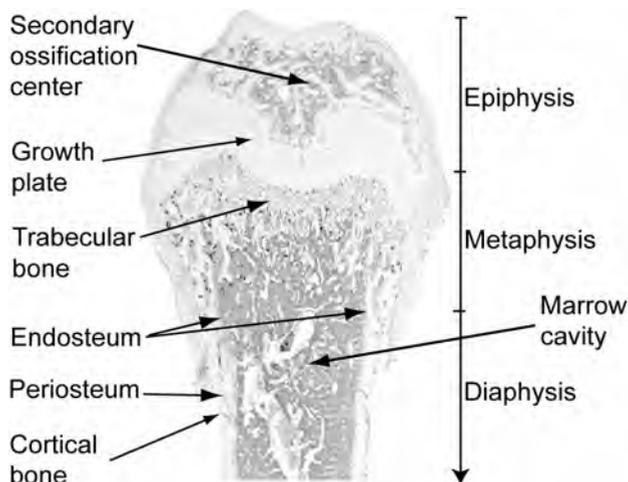
## BONE

### Anatomy and structure of bone

Bone is a complex dynamic tissue that together with cartilage forms the skeletal system. The skeleton provides mechanical support for the body and site of muscle attachment for locomotion as well as protection for vital organs. Bone is highly vascularized, contains the hematopoietic bone marrow and provides a reserve of ions, especially calcium and phosphate. Anatomically, bones can be divided into two major types: flat bones (e.g. calvaria and facial bones, scapula, mandible, sternum) and long bones (tibia, femur, humerus, etc.)

Morphologically, bones can be divided into two forms: cortical (compact) and trabecular or cancellous (spongy). These bones have both structural and functional differences. The cortical bone mainly has a mechanical and protective function, whereas the trabecular bone also has metabolic functions e.g. in calcium homeostasis [1].

A long bone consists of a shaft or diaphysis, and two epiphyses at the endings of the diaphysis (Fig.1). A metaphysis links each epiphysis to the diaphysis. During bone growth, a cartilaginous growth plate is present at the epiphysis-metaphysis interface but after growth, a residual growth line replaces the growth plate. The diaphysis is surrounded by a cylinder of compact bone containing the bone marrow. The epiphyses consist of spongy or cancellous bone covered by a thin layer of compact bone. The periosteum covers the outer surface of the bone (except the articular surfaces and the tendon and ligament insertion sites), whereas the endosteum lines the marrow cavity [2].



**Figure 1. Histology of a long bone**

## **Bone formation and growth**

Two distinct types of developmental processes, intramembranous and endochondral, form flat and long bones, respectively [2].

### **Intramembranous ossification**

Intramembranous ossification occurs during embryonic development and starts with migration and condensation of mesenchymal cells. The cells of these condensations differentiate directly into bone-forming osteoblasts. The first bone is formed as an immature irregular matrix, which calcifies into woven bone. The mesenchymal cells continue to differentiate in the periphery and the matrix becomes vascularized and bone marrow is formed. The woven bone is subsequently remodelled and replaced by mature lamellar bone [3].

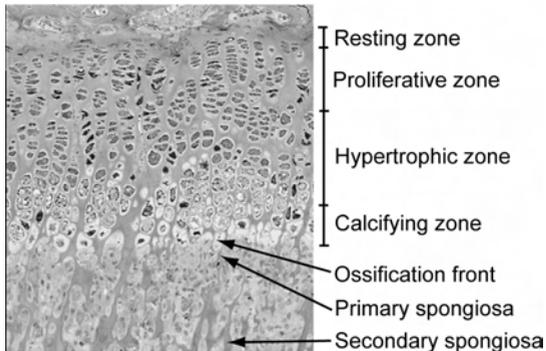
### **Endochondral ossification**

Endochondral ossification is the process by which templates of skeletal cartilage are replaced by bone. The process begins with proliferation and aggregation of mesenchymal cells at the site of the future bone. These mesenchymal cells differentiate into chondroblasts that secrete cartilage matrix that lead to the development of a cartilage model. The chondroblasts become embedded within their own matrix, thereafter called chondrocytes. The first sign of ossification occurs with a change in activity of the perichondrium. Perichondrial cells become osteoblasts, which form a thin bone collar surrounding the future diaphysis. As a result of these changes, the perichondrium is transformed into periosteum. In the mid-diaphyseal region, chondrocytes become hypertrophic, synthesise alkaline phosphatase followed by calcification of the matrix. The hypertrophic chondrocytes undergo apoptosis, matrix breaks down and thus producing a growing cavity. Meanwhile, blood vessels grow into the cavity and get in contact with bone marrow cells. The calcified matrix is partially resorbed and osteoblasts begin to lay down osteoid. This area is called the primary ossification center. In late fetal life and early childhood, secondary ossification centers form in the upper epiphyses in a similar manner [3, 4].

### **The growth plate**

The growth plate (or epiphyseal plate), located below the secondary ossification centre, is the site of longitudinal growth of long bones. Four major zones can be distinguished (Fig. 2). The reserve zone (or resting zone) is a source of mesenchymal stem cells. The proliferative zone is characterized by active proliferation of chondrocytes, arrangement of the cells in vertical stacks and formation of matrix. In the hypertrophic zone the chondrocytes become progressively larger, separate from each other and then undergo apoptosis. In the calcifying zone, the remaining longitudinal septae of cartilage is subsequently calcified. The calcified matrix is partially resorbed by chondroclasts. Blood vessels penetrate the transverse septa and carry osteoprogenitor cells. These cells differentiate and form a layer of woven bone on top of the cartilaginous remnants of longitudinal septae and the primary spongiosa has been

generated. This woven bone is later further remodelled, in which the woven bone and the cartilage remnants are replaced with lamellar bone, resulting in mature trabecular bone called secondary spongiosa. Growth plate inactivation occurs at puberty as a result of increase of estrogen secretion in both men and women, and the growth plates are replaced by trabecular bone leading to cessation of growth [2, 3, 5].



**Figure 2. The growth plate**

### **Regulators of the growth plate**

The transcription factor SOX9 is essential for all phases of the chondrocyte lineage from early condensations to conversion of proliferating chondrocytes to hypertrophic chondrocytes. SOX9 stimulates transcription of a number of cartilage matrix genes e.g. type II collagen and aggrecan. SOX9 deficient mice have cartilage hypoplasia and fewer chondrocytes than normal [6, 7]. Another transcription factor, core binding factor  $\alpha 1$  (Cbfa1 or Runx2) [4] drives the differentiation of proliferative chondrocytes into hypertrophic chondrocytes. Cbfa1-deficient mice have no osteoblasts and also abnormal chondrocyte maturation [8, 9]. They have few hypertrophic chondrocytes that have decreased expression of genes such as osteopontin (OPN) and matrix metalloproteinase (MMP)-13. Indian hedgehog (Ihh) [10], a protein secreted by chondrocytes, regulates proliferation of the growth plate in a paracrine fashion and delays chondrocyte hypertrophy. Ihh also stimulates the expression of parathyroid hormone-related peptide (PTH-rP) [11, 12]. A feedback loop between Ihh and PTH-rP regulates the balance between proliferating and hypertrophic chondrocytes providing a mechanism to ensure active growth plates until puberty [5].

### **Bone matrix**

The bone is composed of an organic matrix, that is strengthened by deposits of inorganic hydroxyapatite  $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$  and also a cellular phase. The organic bone matrix contains type I collagen fibers, non-collagenous proteins, serum proteins and proteoglycans. The most predominant protein of the bone matrix is type I collagen (90%) [13]. The type I collagen fibers consist of triple-helical molecules that are packed into microfibrils with help

of intermolecular crosslinks. The fibrils are further packed into collagen fibers forming a highly ordered structure providing elasticity and flexibility to bone [14]. Collagen determines the toughness of bone, while the mineral confers stiffness. The balance of these two properties of bone determines its overall strength. The cortical bone is calcified 80-90%, whereas the trabecular bone is calcified 15-25% [15].

The extracellular matrix (ECM) of bone contains a relative small amount of the total protein mass of non-collagen proteins, however, together they modulate a wide variety of bone-specific functions including regulation of mineralization, cell adhesion and bone resorption/remodelling. An important sub-group of the non-collagenous matrix proteins are the RGD-containing proteins, such as bone sialoprotein (BSP), OPN, fibronectin, thrombospondin and vitronectin. The RGD sequence is the signature cell-attachment sequence that integrins on the cell-surface bind to, thereby mediating cell-matrix contact. There are also growth factors embedded in the bone matrix, which influence local bone cell activity [13].

### **Bone mineralization**

Bone collagen can be regarded as a scaffold on which mineral deposition occurs. Many of the non-collagenous matrix proteins (NCP) are acidic in character and bind to bone matrix because of their affinity to hydroxyapatite [ $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ]. These non-collagenous matrix proteins actively control mineralization of collagen fibers and crystal growth within osteoid when it is converted to bone. One category of the NCPs is termed the SIBLING (Small Integrin Binding Ligand N-linked Glycoprotein) family and includes OPN, BSP, dentin matrix protein-1 (DMP1), dentine sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE) [16]. The SIBLING proteins are principally found in mineralized tissues e.g. bone and dentine and are secreted into the ECM during the formation and mineralization of these tissues [17]. Moreover, they also display a RGD motif that mediates cell attachment /signaling via its interaction with cell surface integrins.

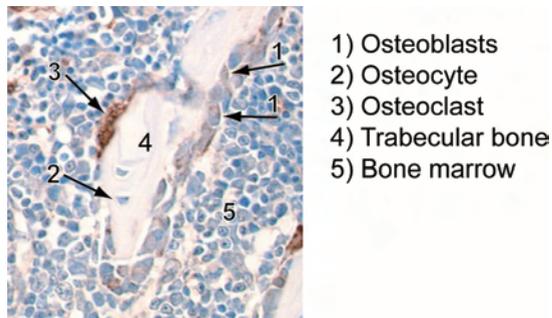
BSP is a glycosylated phosphoprotein highly expressed in mineralized tissues and has been shown to be a potent nucleator of hydroxyapatite formation [18, 19] and as mineral grows on the collagen matrix, it acts as an inhibitor in directing the growth of the crystals [20]. DMP-1 also acts as a nucleator for hydroxyapatite formation [21, 22]. OPN [20, 23, 24] and MEPE [25] are effective inhibitors of apatite formation and growth.

The crystals are transported in membrane-bound extracellular vesicles, known as ECM or matrix vesicles, released from chondrocytes and osteoblasts and distributed along the length of collagen fibers and into the ground substance. The ground substance, composed of glycoproteins and proteoglycans, consists of highly anionic complexes with a high ion-binding capacity and thus participates in the calcification process and in the fixation of hydroxyapatite crystals to the collagen fibers [13].

The ECM vesicles accumulate calcium and phosphate ions, and they also contain enzymes that can degrade inhibitors of mineralization e.g. adenosine triphosphate (ATP), pyrophosphate (PP<sub>i</sub>) and proteoglycans present in the surrounding matrix. They also contain a nucleation core, consisting of proteins and a complex of acidic phospholipids, calcium, and inorganic phosphate, which can induce apatite formation [13, 26, 27].

Alkaline phosphatase (ALP) is an enzyme important for mineralization. ALP hydrolyzes phosphate esters, increasing the local phosphate concentration, enhancing the rate and extent of mineralization. ALP also hydrolysis inhibitors of mineral deposition such as pyrophosphate [28]. Consequently, patients with X-linked hypophosphatasia, a deficiency of alkaline phosphatase, show abnormal bone mineralization [29].

## THE CELLULAR COMPONENTS OF BONE



**Figure 3. Cellular components of bone**

### The osteoblast

Osteoblasts are bone forming cells, responsible for synthesis of the organic matrix, the osteoid, and subsequently control mineralization of the matrix [30]. An equally important function of osteoblasts and preosteoblasts is the regulation of osteoclastic differentiation and resorption via the OPG/RANK/RANKL-system [31, 32]. Osteoblasts derive from mesenchyme-derived osteoprogenitor cells present in the inner layer of the periosteum and the endosteum. Osteoprogenitor cells persist throughout postnatal life as bone-lining cells with the capacity to be reactivated in the adult during the repair of bone fractures and other injuries.

After deposition of the bone matrix, the terminal stages of osteoblast differentiation result in three fates for these cells: they may become inert bone-lining cells, become trapped in the mineralized matrix forming osteocytes or undergo apoptosis.

Osteoblasts are epithelial-like cells with cuboidal shapes, forming a monolayer covering all sites of active bone formation (Fig 3). Osteoblasts display the typical features of cells active

in protein synthesis. Morphologically, they are characterized by a round nucleus, abundant rough endoplasmic reticulum, an extensive Golgi apparatus, and some lysosomal-like bodies [2, 30]. Bone-lining cells are flattened and elongated and are characterized by moderate rough endoplasmic reticulum and Golgi apparatus and stains immunohistochemically positive for intercellular adhesion molecule-1 (ICAM-1) [2, 33].

The differentiated osteoblast is highly enriched in alkaline phosphatase that disappears when the cells become embedded in the matrix as osteocytes. They also synthesize proteins such as type I collagen, osteocalcin, OPN and BSP [13].

The early steps in the differentiation process require the participation of the transcription factors Cbfa1/Runx2 and osterix. Cbfa1/Runx2-deficient mice have a skeleton consisting of cartilage [8]. There is no indication of osteoblast differentiation or bone formation in these mice. In addition, because osteoblasts regulate the formation of osteoclasts, Cbfa1/Runx2-deficient mice lack osteoclasts. Patients with cleidocranial dysplasia have hypertrophic clavicles, delayed ossification of sutures of certain skull bones resulting from an inactivating mutation in the Cbfa1/Runx2 gene [34].

Several growth factors and hormones control proliferation and differentiation of osteoblasts. For instance, members of the bone morphogenic protein (BMP) family and transforming growth factor- $\beta$  regulate development and differentiation of the osteoblast. Osteoblasts and osteocytes have receptors for fibroblast growth factors (FGF), parathyroid hormone (PTH), 1,25-dihydroxyvitamin D<sub>3</sub>, IGF-1, prostaglandins, estrogen and different cytokines [13].

## **The osteocyte**

Osteoblasts differentiate into osteocytes after they are trapped in a lacunae within the mineralized matrix they produce (Fig. 3). It has been suggested that 10-20% of osteoblasts differentiate into osteocytes [35, 36].

These cells form an extensive network of small channels, the canaliculi, which interdigitate through the lamellae and connect neighbouring lacunae. The network of osteocytes provides intracellular communication across gap junctions, thus osteocytes are in direct communication with osteoblasts, osteoclasts and surface-lining cells. Moreover, canaliculi connecting several osteocyte lacunae also provide the cells with nutrients and signaling molecules along the extra cellular environment. It has been suggested that osteocytes play a role as mechanosensors and in local activation of bone turnover [37].

The osteocyte expresses receptors for most of the hormones and cytokines known to be important in bone function, including estrogen receptors, PTH, 1,25-vitamin D<sub>3</sub>, corticosteroids and transforming growth factor- $\beta$  (TGF- $\beta$ ) [36].

Molecules typically expressed in osteocytes are DMP-1 and MEPE. DMP-1 acts as a nucleator of hydroxyapatite formation and is critical for proper mineralization of bone [21, 22]. DMP-1 knockout mice exhibit hypomineralized bone and defective osteocyte maturation [38]. On the other hand, in MEPE knockout mice mineralization is increased suggesting the

opposite function to DMP-1, thus acting as an inhibitor of mineralization [25]. MEPE associate with mature osteoblasts and osteocytes throughout ossification in the skeleton [39] and is thought to control mineralization within the canalicular system.

## **The osteoclast**

### **Introduction**

Osteoclasts are multinucleated cells responsible for bone resorption and mobilization of calcium. The osteoclast precursor is a member of the monocyte-macrophage lineage present in the adjacent bone marrow [40]. Osteoclasts are ultrastructurally characterized by multinuclearity, an abundance of mitochondria and cytoplasmic vesicles and expression of specialized membrane domains on the apical surface facing the bone [2].

Observations on osteopetrotic animals and patients have provided insights into the many factors regulating osteoclastogenesis. Osteopetrosis is, per definition, increased bone mass due to arrested bone resorption, reflecting either failed normal recruitment of osteoclasts or resorptive dysfunction of the differentiated cells [41]. On the other hand, osteosclerosis is caused by enhanced osteoblast activity with failure of osteoclasts to compensate for the increased matrix formation and mineralization.

### **Osteoclastogenesis**

The transcription factors, PU.1 and MTF, acting early in the monocyte-macrophage lineage regulates the development of the hematopoietic precursor cells and the committed precursors to become osteoclasts [42, 43] (Fig. 4). Mice deficient in PU.1 lack osteoclasts as well as macrophages and thus develop osteopetrosis [44, 45].

Initial experiments by Suda [46] revealed that generation of osteoclasts in culture require physical contact of the precursor cells with specific mesenchymal cells such as osteoblasts or marrow stromal cells. It is now clear that macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor- $\kappa$ B ligand (RANKL), both expressed in osteoblast/stromal cells, are essential for osteoclastogenesis. In fact, generation of pure populations of osteoclasts in vitro is achieved by culturing marrow macrophages in the presence of only RANKL and M-CSF [47].

Secreted M-CSF binds to the receptor c-fms, a tyrosine kinase that activates ERK1/2 and PI3-K/AKT. This signaling pathway promotes proliferation of osteoclast precursor cells and survival of the differentiated osteoclast [48]. The critical importance of M-CSF in osteoclast recruitment has been illustrated in the op/op mouse, which lacks functional M-CSF and has osteoclast-deficient osteopetrosis [49].

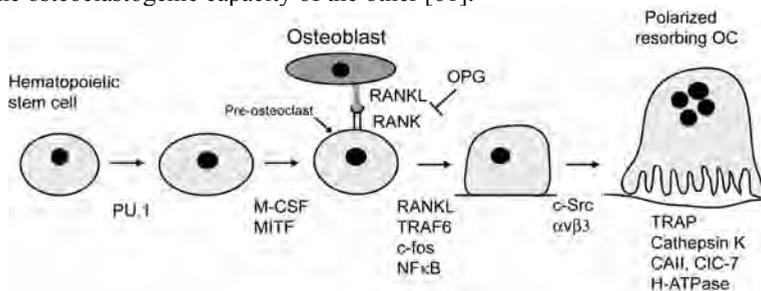
RANKL, a member of the TNF superfamily, is a membrane-residing protein on osteoblasts and their precursors that recognizes its receptor, RANK, on marrow macrophages, which triggers them to attain the osteoclast phenotype [32, 50].

RANKL activity is negatively regulated by osteoprotegerin (OPG), which competes with RANK as a soluble decoy receptor. OPG, like RANKL, is produced by osteoblast lineage cells [51]. OPG overexpression blocks osteoclast production, which leads to osteopetrosis in mice, whereas OPG deletion results in enhanced remodelling of bone and osteoporosis [52]. The disturbance of the OPG/RANKL ratio seems to dictate the rate of bone resorption in a number of pathological states [53].

The signaling downstream of RANK/RANKL is not fully understood. Several TNF receptor-associated factors (TRAFs) can bind to the cytoplasmic domain of RANK as well as other receptors, like the IL-1 receptor [54]. In osteoclastic precursor cells, TRAF6 preferentially binds to RANK and leads to the activation of NF- $\kappa$ B and the JNK/AP-1 pathway, two important transcription factor complexes involved in osteoclastogenesis [55]. Although other receptors such as interleukin (IL)-1R1, CD40, and Toll-like receptors also recruit TRAF6, they do not do so as efficiently as RANK, which may explain their failure to induce osteoclast differentiation alone [56, 57], e.g. IL-1 enhances osteoclastogenesis only in the presence of permissive levels of RANKL [58]. Mice lacking the p50 and p52, the nuclear factor (NF)- $\kappa$ B subunits, also fail to generate osteoclasts and are osteopetrotic [59].

RANKL also promotes osteoclast activation by inducing the mature osteoclast to generate a complex composed of RANK, TRAF6, and c-Src, which the cytokine specifically recruits in the plasma membrane [60]. This event requires organization of fibrillar actin and is mediated via the phosphoinositide-3-kinase (PI3-K)/AKT pathway [60].

Marrow stromal cells are critically involved in the pathogenesis of inflammatory osteolysis such as rheumatoid arthritis where they, when exposed to TNF- $\alpha$ , produce the osteoclastogenic cytokines RANKL, M-CSF, and IL-1. As the inflammatory process becomes more aggressive, TNF- $\alpha$  may promote osteoclast formation by directly stimulating osteoclast precursors without requirement for stromal cells responsive to the cytokine [61-63]. TNF- $\alpha$  and RANKL act synergistically, and minimal levels of one of these cytokines markedly enhance the osteoclastogenic capacity of the other [61].



**Figure 4. Important steps in osteoclast differentiation**

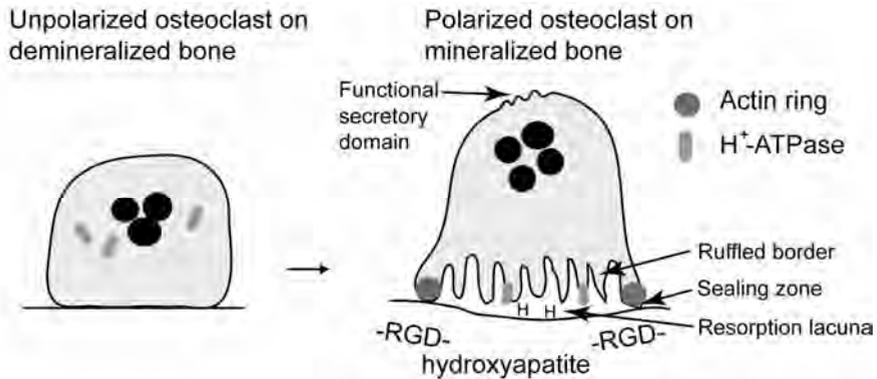
### **Polarization of resorbing osteoclasts**

Non-resorbing osteoclasts are motile cells without distinct membrane domains (Fig. 5). When bone resorption is initiated osteoclasts attach to distinct sites on the bone matrix, become polarized and three specialized membrane domains appear; a sealing zone on the apical side, anchoring the cell to bone matrix, and a ruffled border where the actual degradation of bone takes place as well as a functional secretory domain on the basolateral surface [64-66]. Simultaneously, the cytoskeleton undergoes extensive re-organisation. During this process, the actin cytoskeleton forms an attachment ring at the sealing zone. The sealing zone isolates the resorption lacuna from the extracellular fluid [67], thus creating a compartment at the site of the ruffled border where the decalcification and degradation of the matrix occurs, also referred to as the Howship's lacuna. The functional secretory domain is located at the centre of the basolateral membrane and is in contact with the extracellular fluid, capillaries and also other cells [68-70].

Cell/matrix recognition is mediated by integrins. These  $\alpha/\beta$  heterodimers consist of long extracellular and relatively short intracellular domains. Their function is not only to attach cells to extracellular matrix but also to transmit matrix-derived signals to the cell's interior. The integrins are differentially expressed by osteoclasts during their maturation and  $\alpha_v\beta_5$ , but not  $\alpha_v\beta_3$ , appears on marrow macrophages maintained in the presence of M-CSF [71]. With exposure to RANKL and appearance of the osteoclast phenotype,  $\alpha_v\beta_5$  disappears to be replaced by  $\alpha_v\beta_3$  [72] in the sealing zone. Thus,  $\alpha_v\beta_3$  is the principal integrin expressed by osteoclasts. Mice lacking  $\alpha_v\beta_3$  generate osteoclasts incapable of optimal resorptive activity as their ruffled membranes and actin rings are abnormal *in vivo* [71]. The altered cytoskeleton of the mutant osteoclasts is also apparent by failure of the cell to spread *in vitro* [71]. As a consequence,  $\beta_3^{-/-}$  mice progressively increase bone mass with age. The  $\alpha_v$  family of integrins recognizes the amino acid motif RGD, present in a number of bone matrix proteins such as OPN and BSP. The binding to these ligands activates the integrin by changing its conformation [73]. This outside-in signaling induces a number of intracellular events, one of the most prominent being organization of the actin cytoskeleton. This signalling route, involving PYK-2, c-Src and c-Cbl, controls the formation of the sealing zone [74-76]. The importance of the tyrosine kinase c-Src for osteoclast polarization was demonstrated in c-Src deficient mice [77]. c-Src  $^{-/-}$  mice produce numerous osteoclasts, but they lack ruffled borders and are unable to resorb bone and thus develop osteopetrosis [78]. Interestingly,  $\alpha_v\beta_3$  also regulates osteoclast longevity. The unoccupied integrin transmits a positive death signal mediated via caspase 8, and, therefore, resorptive cells lacking  $\alpha_v\beta_3$  actually survive longer than wild type [79].

It is well known that the osteoclast develop ruffled borders on calcified bone or dentin matrices [80-82] but not on decalcified bone, osteoid or cartilage [82, 83]. Furthermore, disappearance of ruffled borders and prominent reduction in resorption rate were observed after addition of a GRGDS peptide to cultures of osteoclasts on dentine [84].

It has therefore been suggested that hydroxyapatite crystals and bone matrix constituents acting through osteoclast integrins are necessary for the terminal differentiation/activation of osteoclasts [85-89].



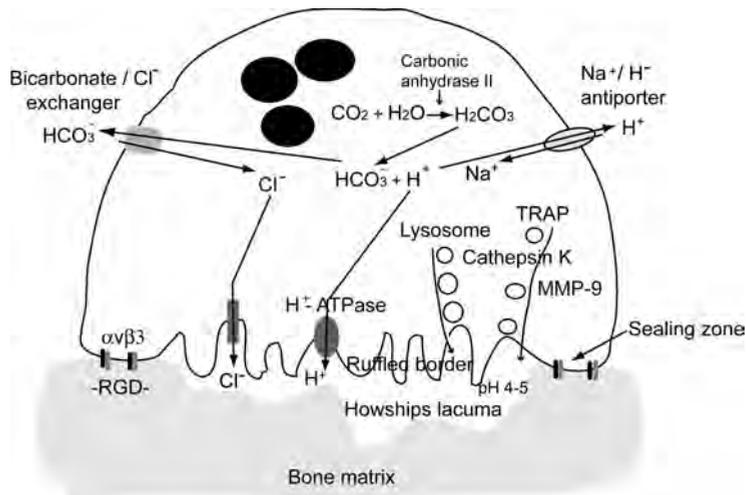
**Figure 5. Unpolarized and polarized osteoclast**

### The ruffled border

The ruffled border is regarded as essential for the resorbing activity of osteoclasts. In resorbing osteoclasts, the ruffled border forms a villous-like structure penetrating into the bone matrix, demarcated by the sealing zone (Fig. 6). The ruffled border is formed by fusion of acidic intracellular vesicles containing a vacuolar-type  $H^+$ -ATPase (V-ATPase), which pump hydrogen ions into the resorption lacuna [90, 91]. The protons are generated in the cytoplasm by the enzyme carbonic anhydrase II [90]. The acidic intracellular vesicles are transported along microtubules [92] to the ruffled border into which they insert mediated by small GTPases [93].

The alkalization of the cytoplasm induced by the massive outward proton transport is balanced by an electroneutral chloride/bicarbonate exchanger [94]. The  $Cl^-$  that enters the cell in exchange for  $HCO_3^-$  [95], is transported into the resorptive lacuna via a chloride channel [96], charge-coupled to the V-ATPase, thus generating HCl, which generate an ambient pH  $\sim$  4.5 in the resorption lacuna [97]. Mice and humans that lack the CIC-7 chloride channel, which is expressed abundantly in the ruffled border membrane [97], develop osteopetrosis [98]. These mice have an abundance of osteoclasts that cannot properly acidify the resorption lacuna and therefore fail to resorb bone. Mutations of the V-ATPase is the most common known cause of osteopetrosis in man [99].

The acid secreted into the resorption lacuna has two functions. Firstly, it mobilizes the mineral phase exposing the organic matrix of bone for degrading proteases, and secondly provides the acidic pH that is required for the action of lysosomal cysteine proteinases, such as cathepsin K [100-104]. In addition to lysosomal cysteine proteases, osteoclasts possess matrix metalloproteinases, such as MMP-9 for collagenolysis [105]. Osteoclasts express high amounts of tartrate resistant acid phosphatase (TRAP) [106, 107]. This enzyme is also secreted during resorption [81].



**Figure 6. Resorptive mechanisms of an osteoclast**

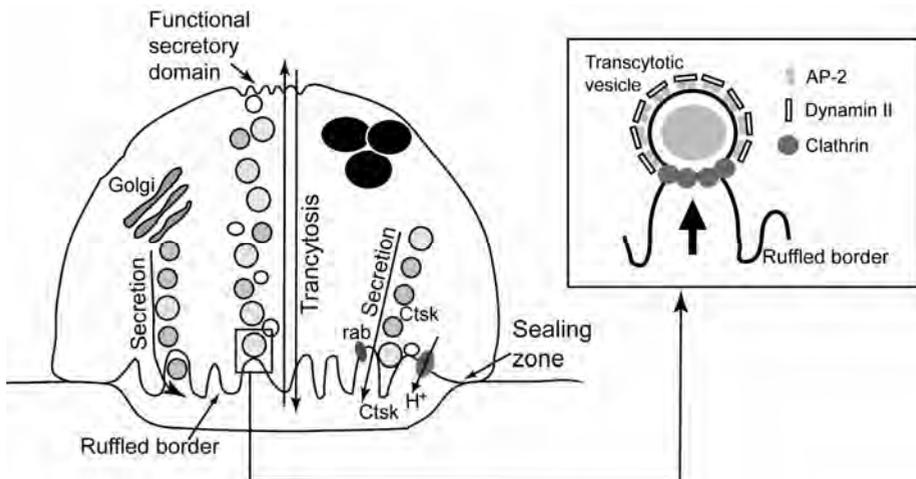
### **Intracellular vesicular transport**

During active resorption the fusion of vesicles forms the ruffled border and both protons and lysosomal proteases and other enzymes are released into the resorption lacuna [108] (Fig. 7). These vesicles originate from the basolateral membrane and from the biosynthetic pathway, i.e. the trans-Golgi network [93, 109, 110]. The ruffled border is also a place for uptake of degradation products [109]. The degradation of collagen and other matrix components will generate high concentrations of peptide fragments in the resorption lacuna of the resorbing osteoclast. The degradation products are endocytosed, transported through the cell and exocytosed through a functional secretory domain on the basolateral surface. This transcytotic route allows osteoclasts to remove large amounts of matrix-degradation products without losing their tight attachment to underlying bone. It also facilitates further processing of the degradation products intracellularly during the passage through the cell and finally delivery through the basolateral membrane into the blood circulation [111, 112]. Both TRAP and Ctsk have been identified in transcytotic vesicles. In addition, it was shown that Ctsk digestion of

TRAP leads to activation of the reactive oxygen species (ROS) generating ability of TRAP [113].

It has been shown that the ruffled border has two subdomains, a peripheral secretory subdomain where membrane fusion takes place and a centrally located uptake zone where degraded matrix is endocytosed [109]. Transferrin endocytosed from the basolateral membrane was delivered to the peripheral ruffled border, colocalized with F-actin, V-ATPase, cathepsin K and Rab7. On the other hand, clathrin, AP-2, dynamin II and the endocytosed bone matrix was concentrated at the central ruffled border [109].

Rab proteins are small GTP-binding proteins that are important regulators of vesicular fusion. Several rab proteins are expressed by osteoclasts but the exact role in vesicular trafficking has been clarified only for some of them. Rab3, Rab7 and Rab9 have been identified in the ruffled border and are potent candidates to regulate vesicular targeting and fusion to the ruffled border [110, 114, 115]. Rab3 regulates exocytosis in a variety of cell types. Several data show that Rab3B/C co-localizes with V-ATPase and pp60c-src in the Golgi membrane fractions within chicken osteoclasts [114]. Rab7, which is a marker of late endosomal compartments in mononuclear cells, is localized at the ruffled border in bone resorbing osteoclasts [110]. Down-regulation of Rab7 with antisense oligonucleotides severely disturbed the polarization of the osteoclasts and the targeting of vesicles to the ruffled border. These impairments caused a significant reduction of bone resorption in vitro [116]. TRAP co-localize with Rab9, a GTPase that has been suggested to participate in the vesicular trafficking between late endosomes and trans-Golgi network [115].



**Figure 7. Vesicular transport and transcytosis in osteoclasts.** Adapted from [117].

### **Functional heterogeneity of osteoclasts**

It has been suggested that functional heterogeneity exist between osteoclasts from different bone sites. For instance, osteoclasts from long bone and calvarial bone differ in the extent they utilize cysteine proteases and matrix metalloproteinases to degrade the organic bone matrix. This has been studied on isolated osteoclasts seeded on bone in the presence or absence of inhibitors for cysteine proteases (CP) and MMPs. It was found that the osteoclastic resorption of calvarial bone depends on activity of both CPs and MMPs, whereas long bone resorption depends on CPs but not on the activity of MMPs [118-120].

Cathepsin K knockout mice have osteopetrotic long bones while their cranial bone structure is normal [121, 122], supporting the view that osteoclasts in different sites are differently dependent on cathepsin K for the resorptive process. Furthermore, significantly higher levels of cathepsin B, cathepsin L and cathepsin K activities were expressed by long bone osteoclasts than by calvarial osteoclasts [120, 123].

When comparing chondroclasts, i.e. cells involved in cartilage breakdown at the epiphyseal/metaphyseal border, with the bone-resorbing osteoclasts in the metaphyseal trabecular bone, differences in ultrastructural features and functional activity were noted [81]. Chondroclasts did not form ruffled borders and clear zones, i.e., well-known ultrastructural bone resorption characteristics, to the same extent as osteoclasts. Instead, chondroclasts tended to express an undifferentiated surface adjacent to the matrix, not structurally different from the basolateral plasma membrane. Moreover, lower levels of secretion of TRAP with concomitant intracellular accumulation of TRAP were apparent in chondroclasts. These results indicate that chondroclasts and osteoclasts differ, not only with respect to location but possibly also by mode of action. The observed differences could reflect the differentiation sequence of these multinucleated cells when associated with different metaphyseal trabecular surfaces [81]. In addition, heterogeneity in structure and function of clasts could be explained by properties of the mineralized matrices they resorb – for example long bones (endochondral) and calvaria (intramembranous) have different molecular compositions [124]. Another explanation could be that the osteoclasts of different sites are derived from different progenitors [125].

### **Bone remodelling and coupling**

Remodelling is the process by which bone is being turned over, allowing the maintenance of the shape, quality and size of the skeleton. Bone remodelling serves to modify the structure of bone in response to changes in mechanical needs and to repair microdamages in bone matrix preventing the accumulation of old bone.

During adulthood, about 10% of bone is replaced each year with complete renewal every 10 years. Bone deposition and bone resorption are ongoing dynamic processes. Osteoclasts and osteoblasts closely collaborate in the remodelling process in what is called a basic

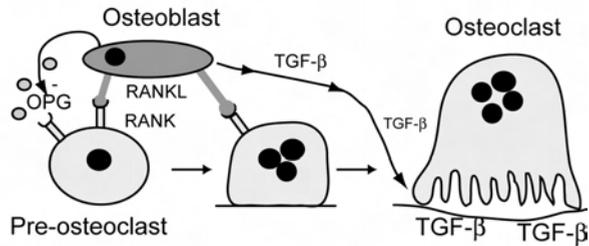
multicellular unit (BMU). The organization of the BMUs in cortical bone and trabeculae bone differs, but these differences are mainly morphological rather than biological. In cortical bone between 2-5% of cortical bone is being remodelled each year, whereas trabecular bone is more actively remodelled than cortical bone due to the much larger surface to volume ratio [126]. The complete remodelling cycle at a specific site takes about 3-6 months [127]. Bone resorption is a faster process compared to bone formation. Bone resorption span weeks, but bone formation take place during months, explaining why resorption sites on the bone surface are less common compared to sites of formation

Remodelling can be divided into four phases; 1) osteoclastic bone resorption, 2) release of osteoclasts and cleaning of the resorption cavity, 3) osteoblast action with osteoid formation, 4) mineralization of the osteoid.

Normally, there is a balance between resorption and formation, and imbalance can have serious consequences. In bone remodelling the term coupling is defined as the strictly regulated replacement of old bone with the same amount of new bone. Molecular communication between osteoblasts and osteoclasts and between bone cells and other bone marrow cells couples precisely bone formation with resorption (Fig. 8). The OPG/RANK/RANKL system allows crosstalk between osteoblastic and osteoclastic cells [128]. Most skeletal disorders are caused by uncoupling in this remodelling process, favouring either bone resorption or bone formation, such as in osteoporosis or osteopetrosis [129].

Both systemic hormones and local factors regulate bone remodelling and the balance between bone formation and resorption. Several cytokines and hormones, including IL-1, estrogen, TNF- $\alpha$ , TGF- $\beta$ , PTH and vitamin D can modulate this system [130].

Osteoclast-to-osteoblast cross-talk occurs mostly through growth factors, such as TGF- $\beta$ , which are released from the bone matrix during resorption [131]. Resorbing osteoclasts can activate TGF- $\beta$  in the low pH environment of their ruffled border [132]. Active TGF- $\beta$  then decreases RANKL and increases OPG expression in osteoblasts [133, 134]. Consequently, fewer RANKL/RANK interactions occur, leading to an inhibition of osteoclast differentiation and activation. On the other hand, TGF- $\beta$  stimulates osteoblast chemotaxis, proliferation/differentiation and osteoid production, thus promoting new bone formation [130, 135, 136].



**Figure 8. Coupling of bone resorption and formation.**

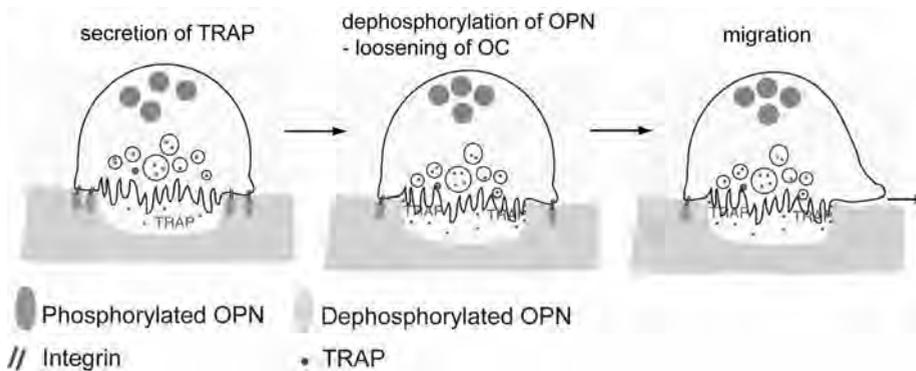
## TARTRATE RESISTANT ACID PHOSPHATASE (TRAP)

Tartrate resistant acid phosphatase (TRAP) is highly expressed and used as a marker for osteoclasts [137, 138] and chondroclasts [81]. This enzyme belongs to the family of purple acid phosphatases, containing a di-iron center as an essential component of the active site [139, 140].

The TRAP enzyme is translated as a 35–37-kDa monomeric proenzyme with low enzymatic activity [141, 142]. Proteolytic processing generates two subunits of 16–18 kDa and 20–23 kDa, linked by an intramolecular disulfide bridge, with enhanced enzymatic activity. This cleaved form is the commonly isolated form from tissues. Based on biochemical studies, the cysteine proteinases cathepsin K [141] and possibly cathepsin L [141, 143] have been suggested to be responsible for proteolytic activation of the enzyme. Cathepsin K co-localizes with TRAP in resorptive compartments of multinucleated osteoclasts, suggesting a role for cathepsin K in the processing of monomeric TRAP [141].

The exact role of TRAP in osteoclasts is not fully understood but studies on TRAP knockout mice showed a mild osteopetrotic phenotype with an extended area of metaphyseal mineralized cartilage and decreased resorptive activity of osteoclasts [144, 145], whereas mice overexpressing TRAP were mildly osteoporotic, with decreased trabecular density in long bones [146].

TRAP protein [81, 82, 147, 148] and enzyme activity [149] can be detected in the matrix adjacent to the ruffled border, suggesting secretion from osteoclasts. Potential functions of the TRAP enzyme are regulation of the biological activity of bone matrix phosphoproteins, e.g. OPN and BSP, by dephosphorylation [150, 151] (Fig. 9). In its phosphorylated state, these proteins are likely to serve as adhesion proteins in the interaction between osteoclasts and matrix, an essential event for the resorption process to begin [152]. *In vivo*, dephosphorylation may be the mechanism to terminate the resorption process before migration, by facilitating the loosening of the cell from the bone surface [150, 153].



**Figure 9. Hypothetical mechanism for extracellular TRAP action in resorbing osteoclasts.**

After secretion into the resorption lacuna the enzyme is probably retrieved by phagocytosis together with matrix remnants [68, 70, 154]. This notion is further supported by the direct demonstration of TRAP, associated with luminal fibrillar material, in lysosome-like vacuoles in osteoclasts [155, 156], in isolated phagosomes [157] and co-localized with internalized bone matrix in large intracellular vacuoles of actively resorbing osteoclasts on bone slices [158]. It is therefore conceivable that the TRAP enzyme exerts its function(s) at several subcellular sites in actively resorbing osteoclasts.

The enzyme can also produce reactive oxygen species (ROS) and ROS generated by TRAP have been suggested to participate in the intracellular degradation of internalized matrix in osteoclasts [159]. A role for TRAP in regulating intracellular vesicular trafficking in osteoclasts has also been suggested since accumulation of intracellular vesicles and an increased ruffled border area was observed in the TRAP-deficient mice [145, 160]

## **PROTEASES RESPONSIBLE FOR BONE MATRIX DEGRADATION**

### **Introduction**

Bone resorption involves the dissolution of bone mineral and the degradation of organic bone matrix. Osteoclasts perform both these processes via their capacity to produce and secrete acid and proteolytic enzymes. Cysteine proteases and metalloproteinases have been identified in osteoclasts and are involved in bone resorption [105, 161]. However, several observations suggest that the degradation of demineralized collagen by cysteine proteases and MMPs is achieved through different mechanisms of action [102, 161]. First, no additive effects of cysteine protease and MMP inhibitors were observed, suggesting that cysteine proteases and MMPs act in series rather than in parallel. Second, MMP inhibitors appeared to act later than

cysteine protease inhibitors suggesting that cysteine proteases and MMPs act at different stages of the resorption cycle.

Bone resorption generate collagen fragments such as carboxyterminal telopeptide of type I collagen (ICTP) and C-terminal cross-linked telopeptide of type I collagen (CTX), which can be quantified in serum and/or urine by using specific immunoassays, and which are used as clinical resorption markers. The generation of ICTP and CTX depends on different collagenolytic pathways. Cathepsin K release large amounts of CTX, whereas the matrix metalloproteinases (MMPs) MMP-2, -9, -13, or -14 release ICTP [162, 163].

## **Cysteine proteases**

Cathepsin K (Ctsk) is a member of the papain-cysteine protease family and has been proven by several lines of evidence to be a key osteoclast protease in bone matrix degradation. Cathepsin K is highly expressed in osteoclasts near the ruffled border membrane as well as in intracellular vesicles of the transcytotic route [113] and has been shown to participate in osteoclast-mediated degradation of the sub-osteoclastic collagenous bone matrix [164]. Cathepsin K exhibit high collagenolytic activity at pH 5-6 and has the ability to cleave the native triple helix of collagen at multiple sites [165]. Once it is cleaved, the triple helix unwinds and becomes available for degradation to any proteinase showing gelatinolytic activity.

Although the main substrates for cathepsin K during bone and cartilage resorption are the fibrillar types I and II collagen [166, 167], the non-collagenous matrix protein osteonectin [164] has been shown to be proteolytically processed by cathepsin K *in vitro*. Also the osteoclast enzyme TRAP [168] was recently shown to be a substrate for Ctsk [141]. Its importance in bone degradation was demonstrated by using selective inhibitors of the enzyme [169-171] or cathepsin K antisense oligonucleotides [172]. Mutations in the human cathepsin K gene have been linked to the human disorder pycnodysostosis, which is an osteopetrotic disorder of variable severity characterized by several features including short stature, a predisposition to bone fractures and dysplasia of membranous bones [173]. Mice overexpressing cathepsin K exhibit a high turnover osteopenia of the metaphyseal trabecular bone [174], whereas mice deficient for cathepsin K show a mild osteopetrotic phenotype with excessive trabeculation of the bone marrow space [121, 122, 174], indicating the importance of cathepsin K in bone resorption.

Other cysteine proteinases, like cathepsins B, C, G and, also have been reported to be present in osteoclasts [103, 175, 176]. Of these, cathepsin L is considered to act extracellularly in subosteoclastic collagen degradation and cathepsin B intracellularly by activating other proteinases [102, 177, 178]. Whether these cysteine proteinases are involved in the modulation of TRAP activity *in vivo* is not known.

## **Matrix metalloproteinases**

Matrix metalloproteinases (MMPs), also called matrixins, are a group of proteinases important for osteoclast function. To date, more than 20 MMPs have been identified. They are extracellular proteinases produced in a latent form and their activation often occurs through proteolytic cleavage. MMPs have been classified and given a name according to several criteria: a prevailing characteristic such as the cleavage specificity (e.g. collagenases, gelatinases, stromelysins), the presence of a given structural domain (membrane-type MMPs) or order of discovery leading to a classification number [179].

The MMPs are able to degrade collagen at neutral pH and also found in areas of resorption are MMP-2, MMP-9, MMP-12, MMP-13 and MMP-14 [180-183]. Addition of inhibitors of MMPs to osteoclasts cultured on bone resulted in large areas of demineralized bone matrix, indicating involvement of MMPs in organic matrix degradation [161]. It seems that the relative importance of cysteine proteases and MMPs varies depending on the bone type, indicating a more important role for MMPs in calvarial bone resorption compared to in long bones [120].

It has been suggested that MMPs may play a role in osteoclast migration since MMP inhibitors completely inhibited recruitment of osteoclasts *in vivo* into the core of diaphysis and osteoclast migration through a collagen matrix *in vitro* [105].

MMP-14 is concentrated at the leading edge of migrating osteoclasts or at the sealing zone of resorbing polarized osteoclasts [184, 185]. MMP-9 is suggested to be involved in vascular invasion of the diaphysis by having a role in solubilization of the cartilage septae in growing long bones. MMP-13 is highly expressed in the hypertrophic chondrocytes and binds to cartilage septae [186]. Knockout mice of both MMP-9 and MMP-13 exhibit increased width of the hypertrophic zone of the growth plate [187, 188]. Another possible mode of action of MMP-9 in osteoclast recruitment is the release of vascular endothelial growth factor (VEGF) and TGF- $\beta$  from the extracellular matrix, thereby inducing osteoclast chemotaxis [189, 190] [191].

## **VITAMIN D**

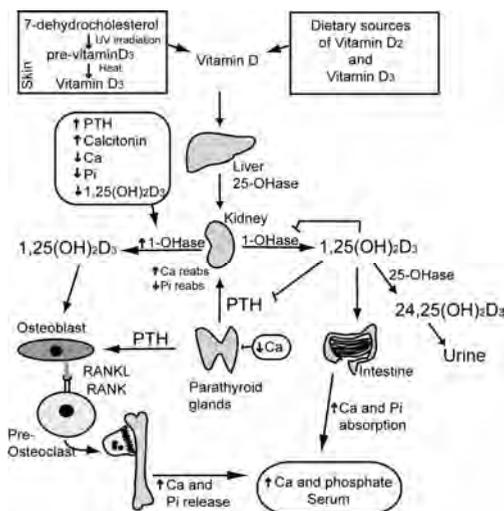
Vitamin D is known as regulator of calcium homeostasis with actions in the intestine, the kidney and bone. It plays a role in systemic as well as intracellular calcium homeostasis of various tissues. The vitamin D receptor is expressed in more than thirty different tissues. Not only calcium- and bone-related genes but also genes regulating the cell cycle, cytokines involved in the immune or haemopoietic system are regulated by calcitriol [192].

### **Vitamin D metabolism**

There are two main forms of vitamin D: vitamin D<sub>3</sub> or cholecalciferol, which is formed in the skin and vitamin D<sub>2</sub> or ergocalciferol, which comes from plants (Fig. 10). In the skin 7-

dehydrocholesterol is converted to previtamin D<sub>3</sub> during exposure to sunlight (UVB light wavelength 290-315 nm) and thereafter converted to vitamin D<sub>3</sub> by a temperature-induced conformational change. When vitamin D<sub>3</sub> is ingested, this fat-soluble compound is incorporated into chylomicrons and absorbed in the lymphatic system and from there enter the circulation. Once the vitamin D<sub>3</sub> enters the circulation, it binds to the vitamin D-binding protein. Vitamin D<sub>3</sub> is transported to the liver, where it is hydroxylated by the enzyme 25-hydroxylase resulting in 25(OH)D<sub>3</sub>. 25(OH)D<sub>3</sub> is the major form of vitamin D<sub>3</sub> in the circulation and the major storage form in the liver. The half life of 25(OH)D<sub>3</sub> in the circulation is 2 weeks and is therefore used for determination of an individuals vitamin D status. 25(OH)D<sub>3</sub> is biologically inert and in order to become active it must be hydroxylated in the kidney by the enzyme 1-hydroxylase to form 1,25(OH)<sub>2</sub>D<sub>3</sub> (calcitriol). Calcitriol enhances intestinal calcium and phosphorus absorption and stimulates the expression of RANKL on osteoblasts which interacts with its receptor RANK on preosteoclasts to induce mature osteoclastic activity, which results in release of calcium and phosphorus [193, 194]. The action of vitamin D<sub>3</sub> is mediated by a nuclear receptor, the vitamin D receptor (VDR), which heterodimerise with the retinoid X receptor and interacts with vitamin D-responsive elements (VDREs) on target genes [193].

The metabolism and synthesis of calcitriol is subject to tight regulation. Hypophosphatemia and hypocalcemia result in increase of renal 1-hydroxylase activity. Parathyroid hormone (PTH) is released in response to hypocalcemia and also stimulates 1-hydroxylase activity. Calcitriol is a regulator of its own production. High levels of calcitriol inhibit 1-hydroxylase and stimulate the formation of a 24-hydroxylase that leads to the formation of the inactive by-product 24,25(OH)<sub>2</sub>D<sub>3</sub> [193, 195].



**Figure 10. Vitamin D metabolism and regulation of calcium and phosphate homeostasis.**  
Adapted from [193].

## **FIBROBLAST GROWTH FACTOR 23 (FGF-23)**

The fibroblast growth factor 23 (FGF-23) was recently discovered to be a hormone involved in renal handling of phosphate and to inhibit phosphate reabsorption and calcitriol production by the kidney. This phosphaturic hormone, which is made predominantly by osteoblasts in bone, appears to have a physiological role as a counter-regulatory hormone for vitamin D. A FGF-23 -bone-kidney axis has been proposed to regulate the mineralization of bone with renal handling of phosphate. On the other hand calcium concentration is controlled by a calcium –PTH-Vitamin D axis. PTH targets the kidney to increase calcium absorption and  $1,25(\text{OH})_2\text{D}_3$  production, and the bone to increase calcium influx.  $1,25(\text{OH})_2\text{D}_3$  stimulates calcium absorption from the intestine which, along with renal and bone calcium, restores calcium to normal.  $1,25(\text{OH})_2\text{D}_3$  regulates FGF-23 production through a VDRE in the FGF-23 promotor [196].

## **HUMAN MODELS**

### **Human rickets and osteomalacia**

Rickets was endemic in Europe and North America during the 19<sup>th</sup> century and during the two first decades of the 20<sup>th</sup> century. The association between lack of sunshine as well as oral vitamin D intake and rickets was recognized in the beginning of the 20<sup>th</sup> century, and around 1920 healing of rickets with sunlight was reported [197]. The encouragement of sensible sun exposure and the fortification of milk with vitamin D resulted in almost complete eradication of the disease [193].

Rickets is a disorder of mineralization of the bone matrix, or osteoid, in growing bone. It occurs during the modelling process; it involves both the growth plate (epiphysis) and newly formed trabecular and cortical bone. Osteomalacia is also a disorder affecting mineralization negatively, but occurs in adults (after growth plate closure) at sites of remodelling. Vitamin D deficiency is the most common cause of rickets. In a vitamin D-deficient state, only 10-15% of dietary calcium and 50-60% of dietary phosphorous are absorbed. Risk factors can be related to insufficient ultraviolet light exposure, such as whole body covering dressing, pigmented skins, age, sunscreen use or insufficient diet, such as vegetarians, breast-feeding for long periods or malabsorption [198-200]. An individual's exposure to sunlight, which is determined by latitude, season and other factors, influences the relative dependence on dietary sources. Fatty fish, fish-liver oil, egg yolk and liver are good dietary sources of vitamin D [193, 195].

### **Pathogenesis of human rickets**

During vitamin D deficiency, intestinal calcium and phosphate absorption are reduced, causing hypocalcemia. The hypocalcemia in turn increase the secretion of PTH from the

parathyroid glands. PTH, (like calcitriol), enhances the expression of RANKL on osteoblasts that increase the production of mature osteoclasts to mobilize calcium stores from the skeleton. In addition, PTH acts on the kidney by increasing tubular reabsorption of calcium and increase phosphate excretion. PTH also stimulates the renal conversion of 25(OH) D<sub>3</sub> to calcitriol which stimulates the small intestine to absorb more calcium and phosphorus [193, 201].

Clinical manifestations of rickets include hypotonia, muscle weakness and, in severe cases, tetany. Weight bearing produces a bowing deformity of the long bones. Biochemical findings includes low or normal serum levels of calcium, low serum levels of phosphorus and elevated serum alkaline phosphate levels, probably reflecting increased bone turnover. The secondary hyperparathyroidism stimulates the kidney to produce calcitriol and thus calcitriol levels are normal or often elevated. Serum PTH levels are elevated when hypocalcemia is present [201].

Hypophosphatemic rickets are found in X-linked hypophosphatemic rickets (XLH), autosomal-dominant hypophosphatemic rickets (ADHR), and tumor-induced osteomalacia (OOM). The common link among them is increased activity of a phosphaturic factor, being the fibroblast growth factor 23 (FGF-23). In these conditions FGF-23 is elevated, resulting in reduction in the phosphate reabsorption by the renal tubuli [200]. Pathologically, high levels of FGF-23 leads to chronic hyperphosphaturia, hypophosphatemia, decreased production of calcitriol, elevated PTH and rickets/osteomalacia in patients with these diseases [202].

Classifications of human rickets and osteomalacia [38, 200, 201, 203, 204];

- *Vitamin D deficient rickets/osteomalacia*  
Caused by insufficient dietary exposure or insufficient ultraviolet light
- *Vitamin D-dependent rickets type I (VDDR type I) or pseudo-vitamin D deficiency rickets*  
Autosomal recessive inheritance, defect in the renal 1-hydroxylase.  
The patients are unable to synthesize calcitriol
- *Vitamin D-dependent rickets type II (VDDR type II)*  
Autosomal recessive inheritance (?), defective intracellular vitamin D receptor
- *X-linked hypophosphatemia (XLH)*  
Impaired FGF-23 proteolysis caused by deficient action of mutant Phex
- *Autosomal dominant hypophosphatemic rickets (ADHR)*  
Inadequate proteolysis of FGF-23 due to proteolysis-resistant mutant FGF-23
- *Autosomal recessive hypophosphatemic rickets*  
Mutations in Dentin Matrix Protein-1 leading by unknown mechanism to increased production of FGF-23
- *Oncogenic osteomalacia (OOM)*  
Increased production of FGF-23 by mesenchymal tumors

## **Human osteopetrosis**

Osteopetrosis was characterized in 1904 by Albers-Schönberg [205] and is caused by failure of the osteoclast to resorb bone. In humans, the osteopetroses include a heterogeneous group of bone disorders characterized by increased bone density. The decrease in osteoclast activity also affects the shape and structure of bone by altering its capacity to remodel during growth. In severely affected patients, the medullary cavity is filled with endochondral new bone, with little space remaining for hematopoietic cells. This abnormality contributes to the brittleness of bone and impaired hematopoiesis in osteopetrosis [41].

The different subforms are classified on the basis of inheritance, age of onset, severity, and secondary clinical features into three major groups: 1) autosomal recessive infantile malignant osteopetrosis, 2) autosomal recessive intermediate mild osteopetrosis and 3) autosomal dominant adult onset benign osteopetrosis. In all these forms, all clinical symptoms are caused by or are secondary to the defect in bone resorption and the consequent increase in bone mass [206]. Thus far, all genes associated with a human osteopetrosis encode proteins that participate in the functioning of the differentiated osteoclast [41, 206].

The three known mutations that have been linked to osteopetrosis in humans cause defects in the acidification phase during bone resorption. The most common of these mutations, found in 50 to 60 % of the osteopetrotic patients, are in the gene encoding the  $\alpha 3$  subunit of the osteoclast vacuolar H-ATPase proton pump. The second most clinically significant mutation affects *CLCN7*, a gene encoding an osteoclast-specific chloride channel [99, 207, 208]. Many of the cases previously described as type II autosomal dominant osteopetrosis are now thought to be related to mutations in *CLCN7* [209]. Carbonic anhydrase II dysfunction is a feature of autosomal recessive osteopetrosis but accounts for only a small proportion of these patients with osteopetrosis [41, 210, 211].

## **Pycnodysostosis**

The important role of cathepsin K in osteoclast function was first suggested by the clinical finding that mutations in this gene caused pycnodysostosis. The human disorder pycnodysostosis is a rare inherited, autosomal recessive, osteosclerotic/petrotic disorder that is caused by mutations of the cathepsin K gene at 1q21 [212, 213]. Clinical manifestation of pycnodysostosis is characterized by short stature, osteosclerosis, acro-osteolysis, spondylolysis, separated cranial sutures with open fontanelles, bone fragility and loss of mandibular angle [214-216]. Histologically, bone from patients with pycnodysostosis is characterized by osteoclasts containing collagen fibrils [216], consistent with a severe defect in bone matrix degradation.

## ANIMAL MODELS

### Animal models of rickets

Animal models with rachitic phenotypes have extensively been used in studies of vitamin D<sub>3</sub> metabolism, phosphate metabolism and osteoclastic resorption mechanisms of poorly mineralized tissues. Although vitamin D<sub>3</sub> is regarded as essential for the development and maintenance of the skeleton, there is little evidence for an active role in bone mineralization. Examples of animal models with a rachitic phenotype are low phosphate, vitamin D-deficient rickets in rats [82], vitamin D receptor knockout mice [217, 218], vitamin D 1-OHase knockout mice [219], DMP1 knockout mice [38], Hyp mice [220] and FGF-23 transgenic mice [221-223].

Studies in growing rats showed that the normal bone formation process was disturbed by introduction of low phosphate, vitamin D-deficient diet resulting in typical features of rickets, including poorly mineralized cartilage and bone matrices. The growth plates were widened and irregular, trabeculae were thickened and cortical bone was thinner. The rachitic osteoclasts were able to establish cell-matrix contact, but the traditional structural features of bone resorption, ruffled border and sealing zones, was poorly expressed and secretion of TRAP but not cathepsin K was limited.

In a similar model, growing rats were kept on a calcium-free diet resulting in increased osteoid volume and a rather irregular trabeculae. However, there was no change in trabecular bone volume and the growth plate width was normal. Osteoclasts in hypocalcemic rats generally exhibited poorly developed ruffled borders but were in contact with both bony and osteoid surfaces [224].

The VDR knockout mice [217, 218], a model of human VDDR type II, showed no defect in development and growth before weaning. However, after weaning the mice exhibited reduced growth, hypocalcemia, hypophosphatemia, elevated serum ALP activity and rickets. There was an increase in the number of osteoblasts without a change in the number of osteoclasts [225]. These findings indicate that 1,25(OH)<sub>2</sub>D<sub>3</sub> is not essential for osteoclast formation *in vivo* or that compensatory mechanisms are activated in this mutant leading to normal osteoclast differentiation. The impaired bone mineralization in the VDR knockout mice was reversed by introducing high calcium diet, indicating that mineralization is not directly dependent on 1,25(OH)<sub>2</sub>D<sub>3</sub> [225-227]. Moreover, the mobilization of calcium from the diet seem to be dependent on the ratio of phosphorus to calcium (Ca:P=2:1) rather than being dependent on only the concentration of calcium in the diet [227].

The osteoblastic marker ALP was found to be increased also in hypocalcemic rats, FGF-23 transgenic mice, DMP1 knockout mice [38], and is known to be increased in human rickets and osteomalacia [193, 200].

Overexpression of FGF-23 in mice cause rickets and osteomalacia [221, 223, 228, 229]. Histology of tibiae displayed a disorganized and widened growth plate and reduced bone mineral density. Transgenic mice were smaller, exhibited decreased serum Pi concentrations and increased urinary Pi excretion. This was explained by decreased expression of Npt2a, the major renal Na<sup>+</sup>/Pi cotransporter in the kidneys of transgenic mice. Serum PTH levels were increased in transgenic mice, whereas differences in calcium and 1,25-dihydroxyvitamin D<sub>3</sub> were not apparent. However 1,25-dihydroxyvitamin D<sub>3</sub> and PTH have also been reported to be decreased [221, 223, 228, 229]. Moreover, as in VDR knockout mice the phenotypic alterations was observed after weaning [229].

### **Animal models of osteopetrosis**

Many genes associated with increased bone density have been discovered using mouse and rat osteopetrotic models (reviewed in [41] [230]).

The following models have been characterized:

- *Knockout mice with defective osteoclast differentiation*  
PU.1, M-CSF, Mitf, RANK, RANKL, c-fos, NF-κB, TRAF-6
- *Knockout mice with defective osteoclast function*  
c-src, cathepsin K, TRAP, β<sub>3</sub>, carbonic anhydrase II, Atp6i (α3 subunit of V-ATPase), CIC-7
- *Transgenic mice with defective osteoclast differentiation*  
OPG
- *Spontaneous mutations in mice and rats*  
Ia/ia, op/op, grey-lethal, tl/tl (mutation in the CSF-1/M-CSF gene)

### **Tartrate-resistant acid phosphatase (TRAP) –deficient mice**

Hayman et al. have generated a TRAP knockout mouse model [144]. In TRAP-deficient mice the long bones were shorter and had thicker cortices but apparently normal tooth eruption and skull plate development, indicating a defect in endochondral ossification. The epiphyseal growth plates in six- to eight-week old mutant mice were widened with delayed mineralization of cartilage. Furthermore, the columns of chondrocytes were disorganized with increased retention of thicker trabeculae. The intramembranous bones of the skull showed increased density at all ages examined, indicating defective osteoclastic bone turnover. Increased mineralization occurred in the long bones of older animals, reflecting a mild osteopetrosis caused by reduced osteoclast modelling activity. Osteoclasts from TRAP-knockout mice were found to differentiate and form resorption pits in vivo, but they resorbed less efficiently compared to cells from wild type littermates [144]. The authors concluded that TRAP is required for normal mineralization of cartilage in developing bones; and also maintains integrity and turnover of the adult skeleton by a critical contribution to bone matrix resorption.

The rate of collagen synthesis and degradation were significantly greater in bones from TRAP<sup>-/-</sup> mice compared with wild type [231]. At 8 weeks, there was an increase in the intermediate cross-links but no significant difference in animals aged 6 months. There was a significant increase in mature cross-links at both ages. A significant increase in production of both pro and active MMP-2 was observed. The biomechanical properties were investigated using a three-point bending technique. The bones from TRAP<sup>-/-</sup> mice required a higher ultimate stress and yield stress in order to break compared with wild type. The stiffness of elasticity was higher at 8 weeks in the TRAP<sup>-/-</sup> compared to wild type mice, however, at 6 months the elasticity had decreased. Thus, both synthesis as well as degradation of collagen were increased in mice at 8 weeks and 6 months of age when TRAP was absent, showing that TRAP has an important role in the metabolism of collagen [231].

### **Lysosomal acid phosphatase (LAP)/ TRAP –double deficient mice**

Two lysosomal acid phosphatases are known to be expressed in cells of the monocyte/phagocyte lineage: the ubiquitously expressed lysosomal acid phosphatase (LAP) and the tartrate-resistant acid phosphatase-type 5 (Acp5). In LAP knockout mice, abnormalities of bone structure were apparent in mice older than 15 months, resulting in malformation of the lower thoracic vertebral column. LAP knockout mice had higher than normal levels of TRAP activity, suggesting that LAP and TRAP may have overlapping functions. Moreover, mice deficient in LAP have excessive lysosomal storage in podocytes and tubular epithelial cells of the kidney as well as microglia, ependymal cells and astroglia within the nervous system [232].

Suter et al. [233] generated LAP/TRAP double deficient mice. These mice displayed marked alterations in soft and mineralized tissues. They were characterized by a progressive hepatosplenomegaly, gait disturbances and exaggerated foreshortening of long bones. Moreover, these animals were distinguished by an excessive lysosomal storage in macrophages of the liver, spleen, bone marrow, kidney and by altered growth plates. OPN was accumulated adjacent to actively resorbing osteoclasts of TRAP- and LAP/TRAP-deficient mice. Vacuoles in the osteoclasts contained fine filamentous material, crystallite-like features, as well as OPN. Bone extracts from these mice were unable to dephosphorylate recombinant OPN, confirming that TRAP is important for processing of this protein. Isolated osteoclasts from the LAP/TRAP-deficient mice showed a smaller reduction in resorption compared with osteoclasts deficient in only TRAP.

They concluded that for several substrates LAP and TRAP can substitute for each other and that these acid phosphatases are essential for processing of non-collagenous proteins, including OPN, by osteoclasts. However, a single deficiency of TRAP but not LAP led to phenotypic alterations in bone and cartilage.

## **Cathepsin K –deficient mice**

Cathepsin K, which has high proteolytic activity and localizes primarily in osteoclasts, was discovered in 1995. This first tissue-specific cathepsin was associated with pycnodysostosis, a human genetic osteopetrotic disorder observable as an osteopetrotic phenotype in cathepsin K-deficient mice. Up to now five independent groups [121, 174, 234-236] have generated cathepsin K-deficient mice. Cathepsin K-deficient mice survive and are fertile, but display an osteopetrotic phenotype of the long bones and vertebrae with increase in trabecular bone volume, trabecular thickness and trabecular number [121]. Moreover, femurs of Cathepsin K-deficient mice exhibit a greater cortical area and thickness, normal strength, but a high degree of brittleness [235], possibly due to extensive areas of woven bone and intracortical resorption spaces within the disorganized tissue [235]. In addition, bone formation indices were altered in *Ctsk*<sup>-/-</sup> mice, with significant increases in mineral appositional rate, but not in bone formation surface, suggesting increased osteoblast activity but not recruitment due to *Ctsk* deficiency.

Cathepsin K-deficient osteoclasts exhibit a modified morphology: their resorptive surface was poorly defined with a broad demineralized matrix fringe containing undigested fine collagen fibrils; their ruffled borders lacked fingerlike foldings, and they were devoid of collagen fibril-containing cytoplasmic vacuoles and actin rings were partially disrupted. Moreover, the cathepsin K-deficient osteoclasts harboured large vesicles containing native type I collagen often found in the upper parts of the osteoclasts, indicating impaired proteolytic processing of organic bone matrix in the absence of cathepsin K [121, 232].

The cathepsin K-deficient osteoclasts were fully differentiated but their resorptive activity was impaired, as indicated by decreased levels of the resorption markers ICTP and CTX in the media of cathepsin K-deficient osteoclasts cultured on bone slices [174]. Studies of resorption on dentine sections showed that cathepsin K-deficient osteoclasts generated pits with larger areas, but with smaller volumes and depths than controls [121]. Osteoclasts were able to demineralize the bone normally via functional vacuolar ATPase activity, but the primary defect in these mice was seen in the resorption and endocytosis of the organic phase of the bone matrix [121].

Kiviranta et al. [174] reported several mechanisms attempting to compensate for *Ctsk* deficiency in Cathepsin K-deficient mice. An increase in RANKL/ OPG ratio favoring osteoclastogenesis and also an increased number of osteoclasts [174, 235] in trabecular bone was observed. In addition, expression of mRNAs of osteoclastic enzymes (MMP-9, TRAP) and osteoblastic proteases (MMP-13, MMP-14) were increased in *Ctsk*<sup>-/-</sup> mice compared to controls. They concluded that impaired osteoclastic bone resorption in *Ctsk*<sup>-/-</sup> mice results in

activation of osteoblastic cells to produce increased amounts of other proteolytic enzymes and RANKL in vivo.

List of generated cathepsin K knockout strains:

- Saftig et al. [122] Outbred 129SVJ-C57BL/6J
- Gowen et al. [121] C57BL/6J-BALB/c
- Kiviranta et al. [174] Outbred C57BL/6J
- Li et al. [235] C57BL/6J
- Chen et al. [236] Inbred 129/Sv and inbred C57BL/6J

Not all characteristics of the human pycnodysostosis phenotype were found in the first four cathepsin K knockout C57BL/6J outbred strains, therefore Chen et al. [236] generated two cathepsin K-deficient inbred strains of 129/Sv or C57BL/6J background. Examination of the two strains showed that only the 129/Sv background cathepsin K-deficient mice exhibited many of the characteristics of the human pycnodysostosis-like phenotype e.g. acro-osteolysis, spondylolysis, and decreased calvarial thickness. Moreover, the cathepsin K-deficient inbred 129/Sv strains showed considerably more osteoclasts than the C57BL/6J strain. These osteoclasts lacked normal apoptosis and senescence, which may function as a feedback mechanism controlling osteoclast numbers in bone homeostasis.

# PRESENT INVESTIGATION

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## AIMS OF THE THESIS

The overall aim of the thesis was to correlate the ultrastructural morphology of osteoclasts with function using osteopetrotic and rachitic animal models

### Specific aims:

- To study the events during initiation and progression of resorption with focus on correlating ultrastructural characteristics of osteoclasts with secretion of TRAP, cathepsin K and MMP-9.
- To further characterize the relation between osteoclast polarization and secretion of proteolytic enzymes in hypomineralized bone.
- To study the role of mineral on the osteoclast-mediated degradation of bone matrix.
- To investigate consequences of genetic depletion of TRAP on the phenotype of bone resorbing osteoclasts and bone.
- To investigate the involvement of cathepsin K in expression, proteolytic processing and vesicular transport of TRAP in osteoclasts in vivo.

## COMMENTS ON METHODOLOGY

### Quantitative morphology - stereology

Stereology is a collection of mathematical methods that allows quantification of three-dimensional structures from measurements of a structure on two-dimensional sections. The need for quantitative information is of particular importance when evaluating phenotypes of animal models such as knockout- and transgenic mice, since reports based on qualitative findings from tissue sections are not statistically reliable.

The method uses microscopical techniques and is based on the principles of histomorphometry e.g quantitative histology based on stereology, which can be used to calculate areas and volumes, as well as numbers and areas of specific cell types on two-dimensional sections. Generally the quantity of the structure is estimated in relative terms, ie densities. The density is defined as the quantity per volume, area or length within a defined space - the reference space. Proper sampling is fundamental, since these methods are based on mathematical assumptions of geometric probability and statistics [237].

In our studies, we used animal models to investigate in vivo effects on osteoclasts and trabecular bone. The volume density of trabecular bone or osteoclasts within the metaphysis in the bone was estimated by point counting using a square lattice from micrographs of bone tissue sections.

For instance, the volume density ( $V_v$ ) of trabecular bone was calculated as the ratio of the number of test points ( $\Sigma P$ ) falling on the trabecular bone to all the points falling in the metaphysis (reference area).

$$V_v (\text{trabecular bone}) = \frac{\Sigma P (\text{trabecular bone})}{\Sigma P (\text{reference area})}$$

Other techniques for analysis of the composition of bone tissues are DEXA (dual X-ray absorptiometry) and pQCT (peripheral quantitative computerized tomography). With the DEXA technique it is possible to measure intact bones, either in vivo or dissected, in two dimensions, giving the total bone mineral content, length and area of the bones analyzed. The pQCT technique measures the bone in three dimensions, either dissected or in vivo, giving for example the bone mineral content of trabecular and cortical bone as well as calculated trabecular volumetric bone density. A significant correlation between the two techniques measuring the trabecular density (pQCT and histomorphometry) has been shown [238].

## **Immunohistochemical markers for osteoclasts**

TRAP is often used as a histochemical marker for osteoclasts, however TRAP is expressed by several cell types derived from differentiation along the macrophage lineage [239], thus both pre-osteoclasts, certain activated macrophages and dendritic cells along with osteoclasts will be labelled using TRAP antibodies in immunohistochemistry. Since cathepsin K does not appear to be as broadly expressed as TRAP among myeloid cells, cathepsin K was therefore considered a more selective immunohistochemical marker for osteoclasts in stereological studies.

## **Antibodies recognizing TRAP**

TRAP is translated as a single monomeric polypeptide, but the protein isolated from different tissues commonly exist as a disulfide-linked dimeric (two-subunit) structure with an N-terminal fragment of 20-23 kDa joined to the 16-18 kDa C-terminal fragment. Two different antibodies recognizing monomeric and total i.e. both monomeric and dimeric TRAP, was used in the studies of the thesis.

- The antibody recognizing *total TRAP* was raised in rabbits with recombinant monomeric rat TRAP as antigen. This antibody recognize both the monomeric and two-subunit forms of bone TRAP [141].
- The second antibody selectively recognizing the *monomeric*, uncleaved *TRAP* was raised in rabbits against a peptide derived from the mouse TRAP sequence (<sup>146</sup>DDFASQQPKMPRDLGVA<sup>162</sup>) as the immunogen [239]. This sequence contains the major part of an exposed loop domain in TRAP, which is excised by cathepsin K to convert the monomeric to the dimeric form. This antibody thus recognizes only the monomeric form of TRAP.

## **Immunogold technique**

Immunohistochemistry at the ultrastructural level was used to quantify concentrations of proteins in defined compartments inside and outside the osteoclast. Ultrathin sections were incubated with antibodies towards the protein of interest (TRAP, Ctsk or MMP-9). Bound antibodies were detected by protein A conjugated to 10 nm colloidal gold particles. Micrographs were obtained by electron microscopy. The concentrations of immunolabelling represented by the electron-dense gold particles were estimated by calculating the ratio of counted gold particles and measured areas of selected compartments.

## RESULTS AND DISCUSSION

### Rachitic animal models (Paper I & II)

#### Vitamin D-deficiency rickets and healing in rats (Paper I)

In this study healing of rachitic lesions in rats was used as a model for studying the activation sequence of resorbing osteoclasts. Rickets was induced in 3-week-old weaned male Sprague-Dawley rats by feeding a vitamin D-free diet containing low (0.2%) content of phosphate and high (2.2%) calcium. After 4 weeks on this diet, a standard rat diet containing 0.75% phosphate, 0.98% calcium and 1500 U of vitamin D<sub>3</sub> per kg was provided and the animals were sacrificed after 48 and 72 h. In ultrastructural analyses, the two groups of healing animals were compared with previous results from rickets [82].

The rats exhibited a disturbed ossification of the metaphysis typical of rickets, however, qualitative observations showed a successive normalization of the epiphyseal/metaphyseal border after introducing normal food, evident already after 48 h. Quantitative ultrastructural analysis showed that the characteristic features of actively resorbing cells, e.g. ruffled borders and clear zones, were only present in a small proportion (< 10%) of the rachitic osteoclasts. Instead rachitic osteoclasts expressed intermediate or undifferentiated zones, i.e. signs of incomplete or absent polarization, respectively. At the ultrastructural level, healing was characterized by development of ruffled borders and clear zones in the altered osteoclasts that reached normal levels at the 48 h healing time-point. These results indicate that mineralization of bone matrix (with mineral-associated proteins) is required for osteoclast polarization.

Analyses by the immunogold technique revealed that cathepsin K was secreted at the ruffled border area at all time points. In contrast, the levels of tartrate-resistant acid phosphatase (TRAP) were low both between ruffles and in the outside matrix adjoining the ruffled border in polarized clasts in rickets and at the early (48 h) healing time-point, but were increased at the latest (72 h) healing time-point. These results combined with the quantitative ultrastructural analyses suggest that restoring mineral balance, i.e. healing of rickets, normalize osteoclast structure and secretion of cathepsin K faster than secretion of TRAP. Thus, although TRAP and cathepsin K are confined to the same osteoclast populations the secretion of TRAP and cathepsin K seems to be differentially regulated during the resorption process.

The expression of TRAP and cathepsin K at the mRNA level, as well as protein expression and activity of TRAP, were not altered during the healing sequence. Thus, osteoclast polarization does not affect transcription or synthesis of TRAP or cathepsin K.

Cathepsin K was shown to be accumulated in between and associated with the ruffles, while TRAP was mainly located in the matrix outside the ruffled border, more distant from the cell.

The consequence of this distribution should be that collagen degradation by cathepsin K occurs in or very close to the ruffled border, coincident with dissolution of hydroxyapatite due to local acidification in this area. The accumulation of TRAP in the matrix indicates a role in bone resorption, possibly related to dephosphorylation of matrix proteins [150]. Moreover, the temporal sequence of TRAP secretion during healing supports the notion that the enzyme may act extracellularly as a regulator of the resorption process, e.g. by dephosphorylating proteins such as osteopontin (OPN) involved in adhesion and activation of osteoclasts, rather than directly participating in the degradation of the bone matrix.

In conclusion, regulation of TRAP and cathepsin K at the transcriptional level is not affected by the polarization of the osteoclast. Whereas secretion of cathepsin K is associated with onset of the resorption process, extracellular TRAP appears to act at a later stage during the resorption sequence. Moreover, since TRAP is not secreted in polarized osteoclasts at the early healing time-point, a role for this enzyme in the formation of ruffled borders appears unlikely.

### **FGF-23 transgenic mice (Paper II)**

Fibroblast growth factor-23 (FGF-23) is an important determinant of phosphate (Pi) homeostasis and vitamin D metabolism acting by inhibition of renal Pi reabsorption and by suppression of 1,25-dihydroxy vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) production in the kidney [204, 240]. Overexpression of FGF-23 in mice causes hypophosphatemia, reduced 1,25(OH)<sub>2</sub>D<sub>3</sub> levels and rickets [221, 223, 228]. The bones exhibit widened and disorganized growth plates and reduced bone mineral density characteristic of rickets [221, 223].

Hypophosphatemic transgenic (tg) mice over-expressing FGF-23 under the control of the  $\alpha$ 1(I) collagen promoter in osteoblasts were used in this study as an *in vivo* model to examine the relation between osteoclast polarization, secretion of proteolytic enzymes and resorptive activity.

In the long bones of these tg mice, the mRNA expression levels of the osteoblast differentiation marker Runx2 and mineralization-promoting proteins alkaline phosphatase (ALP) and bone sialoprotein (BSP) were increased, whereas expression of  $\alpha$ 1(I) collagen, osteocalcin, dentin matrix protein 1 and OPN were unchanged compared to wild type (wt) mice. Total alkaline phosphatase was 2.5-fold increased in the serum of FGF-23 transgenic compared to wild type mice, and the labeling for BSP protein in metaphyseal bone, visualized by immunohistochemistry, appeared more intense on the bone surfaces of the FGF-23 transgenic compared to wild type mice. The data indicate selective activation of mineralization-promoting osteoblast genes (ALP, BSP) in this model of rickets, possibly regulated by the low phosphate concentrations in the extracellular fluids. Whereas increased serum ALP activity is characteristically increased in rickets [193, 200], the potential value of BSP as a novel serum marker for diagnosis of rickets needs to be further explored. The selective up-regulation of ALP and BSP among several osteoblast markers also underscores

the potential risk or limitation of interpreting a change in e.g. ALP, either as a histochemical or serum osteoblast marker, as reflecting a change in osteoblast numbers or overall activity, respectively.

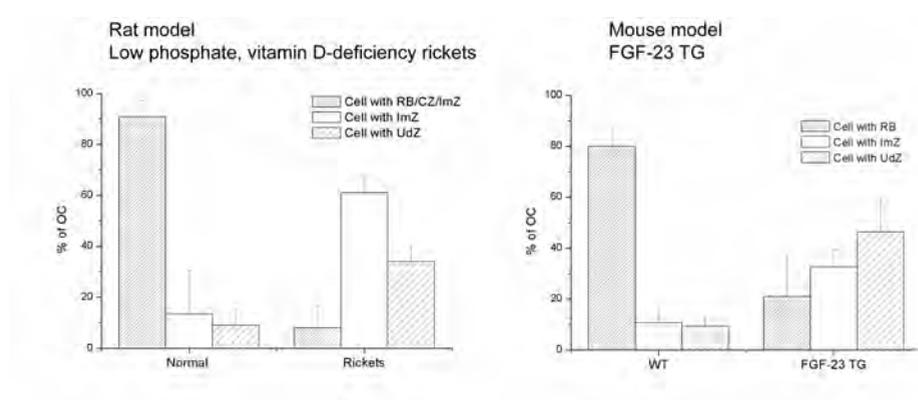
The number of osteoclasts was not different in tg compared to wt mice, as determined by histomorphometry, normal serum levels of TRAP 5b activity as well as unaltered mRNA expression levels of TRAP and cathepsin K. These data indicate that osteoclast differentiation and/or survival is not affected in rickets. Of the osteoclast marker genes analyzed only matrix metalloproteinase-(MMP-) 9 was significantly increased.

At the ultrastructural level, the majority of osteoclasts in FGF-23 transgenic mice exhibited undeveloped resorptive structures indicative of osteoclast dysfunction. Hence, only around 20% of osteoclasts in FGF-23 transgenic mice compared to 80% in control mice developed morphological signs of proper polarization i.e. ruffled borders and clear zones. It has previously been demonstrated that the osteoclast develop ruffled borders on calcified bone or dentin matrices [80-82] but not on decalcified bone, osteoid or cartilage [82, 83]. It has therefore been suggested that hydroxyapatite crystals and bone matrix constituents are necessary for the terminal differentiation/activation of osteoclasts [85-89]. The present study supports this view in a structural, but not functional context. In spite of an unchanged number of osteoclasts and altered polarization of the osteoclasts, elevated serum concentrations of C-terminal telopeptide of collagen I (CTX) were indicative of increased bone matrix degradation in the tg mice. The data in **paper I** suggested a coupling of cathepsin K secretion to ruffled border formation. This interpretation is not in line with the findings in the FGF-23 tg mice where the majority of osteoclasts lacked ultrastructural morphological signs of proper polarization but secreted both cathepsin K and MMP-9 at levels comparable to osteoclasts with ruffled borders. Therefore, mineralization of bone matrix appears essential for inducing osteoclast polarization (consistent with **paper I**) but not for secretion of osteoclast proteases.

Thus, increased CTX serum levels were not due to increased secretion of collagenolytic proteases in osteoclasts from FGF-23 transgenic mice. Instead, release of CTX by freshly isolated osteoclasts was increased on demineralized compared to mineralized bovine bone slices, indicating that the mineral component limit collagen degradation. It therefore appears that in hypomineralised bone such as in rickets, the proteolytic enzymes secreted by osteoclasts are not limited by a mineralized matrix resulting in an increased rate of collagen degradation as a consequence.

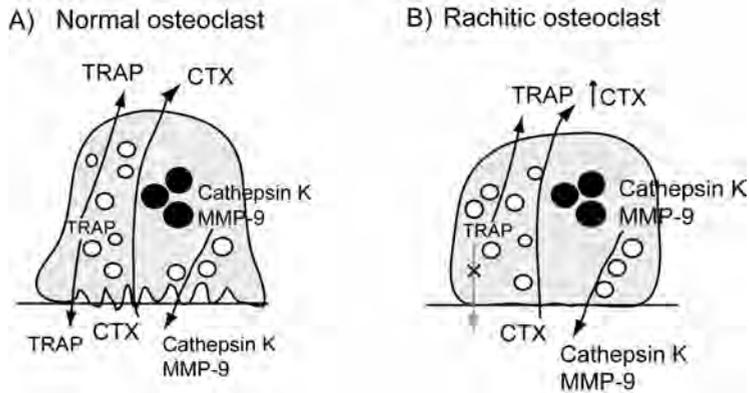
The data thus implicate the importance of ruffled borders for acidification and subsequent demineralization of the bone matrix but not required for matrix degradation. The data collectively provide evidence that osteoclasts, despite absence of ruffled borders, effectively participate in the degradation of hypomineralized bone matrix in rachitic FGF-23 tg mice.

Thus, our data does not concur with the widely accepted notion that the activation of osteoclasts for bone resorption is necessarily coupled to polarization of the cell, at least not in hypomineralized bone. Furthermore, the presence and relative area of ruffled borders are often regarded as indicators of the resorptive capacity of individual osteoclasts, while the present study indicate that such a correlation may be restricted only to osteoclasts associated with mineralized matrix surfaces and may not be generally valid as an ultrastructural morphological measure of osteoclast activity.



**Figure 11. Comparison of the ultrastructural morphology of osteoclasts in untreated rats [81] and mice (paper II) with the dietary rachitic rat model [82] and the FGF-23 tg mouse model (paper II).**

The ultrastructural morphology of osteoclasts in the two animal models was similar (Figure 11). In normal rat and wild type mice the majority of osteoclasts were polarized, thus expressing ruffled borders and clear zones. In rachitic animals from both models, the majority of osteoclasts were either expressing an undifferentiated zone or an intermediate zone, indicating a key role for mineralization of bone matrix to induce osteoclast polarization.



**Figure 12. Hypothetical model of secretory processes in normal and rachitic osteoclasts**

When studying the secretion of proteolytic enzymes from rachitic osteoclasts, the data suggest that cathepsin K (**paper I and II**) and MMP-9 (**paper II**) are secreted normally into the resorption lacuna (Figure 12B). TRAP was normally secreted into serum in rachitic mice (**paper II**) but was not secreted into the bone matrix until after 72h with normal diet (**paper I**), indicating that TRAP is secreted after the osteoclast has developed ruffled borders and has initiated degradation of the bone matrix. CTX, a serum marker for collagen degradation, was higher in rachitic animals compared to wt (**paper II**), indicating that the osteoclast degrades collagen I more efficiently when the fibers are not mineralized.

## Osteopetrotic animal models (Paper III & IV)

### TRAP-deficient mice (Paper III)

In this study, mice with an inactivated TRAP gene developed by Hayman et al [144] was used to assess the influence of TRAP on skeletal morphological phenotype and osteoclast structure and function in young and adult mice. TRAP-deficient mice displayed alterations of the epiphyseal growth plates as was evident by increased height with disorganized columns of chondrocytes, in particular affecting the zone of hypertrophic chondrocytes, consistent with a disturbance of chondrocyte maturation and chondroclastic resorption at the epiphyseal/metaphyseal junction. In addition, TRAP<sup>-/-</sup> mice expressed an early onset osteopetrotic/osteosclerotic bone phenotype, manifest already at 4 weeks of age, when the relative volume of trabecular bone (BV/TV) in distal femur metaphysis was increased compared to wild-type mice. Thus, TRAP deficiency is associated with increased trabecular bone volume as in osteopetrosis or osteosclerosis, and disturbed maturation of epiphyseal cartilage.

The differentiation of osteoclasts was apparently normal, since the number of osteoclasts was increased in both young and adult TRAP-deficient mice to the same extent as the trabecular bone. The osteoclasts in TRAP-deficient mice were however less active in terms of degradation or release of the resorption marker C-terminal type I collagen cross-linked peptide (CTX), indicative of an intrinsic defect. These data indicate that TRAP-deficient mice are osteopetrotic since an increased number of osteoclasts together with unchanged collagen degradation indicate decreased resorptive activity per osteoclast. However, increased expression of Cbfa1 and BSP indicate activation of osteoblasts suggesting a mixed osteopetrotic/osteosclerotic phenotype.

Ultrastructural morphometry disclosed that osteoclasts from TRAP deficient young mice exhibited a normal total cell area but increased relative area of ruffled borders. This could be part of a compensatory mechanism to increase resorption in a situation where a critical factor i.e. TRAP is not expressed. Moreover, accumulation of medium-sized vacuoles in the cytoplasm of TRAP-deficient osteoclasts indicates a role for TRAP in the regulation of osteoclast intracellular membrane traffic. However, since cathepsin K was detected at normal levels in the ruffled border area and matrix in TRAP<sup>-/-</sup> mice, accumulated vesicles were not likely derived from the secretory pathway. Additionally, the decreased resorptive activity per osteoclast detected in this mutant strain could not be explained by impaired secretion of cathepsin K to the resorption lacuna.

In summary, the resorptive defect in TRAP-deficient osteoclasts is reflected by a disturbance at the level of ruffled borders and intracellular transport vesicles. Consequently, accumulation of vesicles in the cytoplasm of mutant osteoclasts indicates a novel function for TRAP in

modulating intracellular vesicular transport in osteoclasts. Whether this action of TRAP is due to the secreted or intracellular forms, or requiring the enzymatically active, dimeric variant of the protein could not be distinguished using this model.

#### **Cathepsin K-deficient mice (Paper IV)**

Recent biochemical studies have indicated the possibility that cysteine proteases, in particular cathepsins K and L, could be involved in the generation of enzymatically active TRAP by converting the monomeric biosynthetic form to the dimeric form by proteolytic processing in an exposed loop domain [141, 241].

In this study, 4-week old *Ctsk*<sup>-/-</sup> mice were used to delineate a role for cathepsin K in TRAP processing in osteoclasts *in vivo*. The absence of cathepsin K in osteoclasts was associated with increased expression of TRAP mRNA, monomeric TRAP protein and total TRAP activity. Interestingly, proteolytic processing of TRAP was not abolished but prematurely arrested at an intermediate stage without changing enzyme activity, a finding confirmed with RANKL-differentiated osteoclast-like cell line RAW264.7 treated with the cysteine proteinase inhibitor E-64. Thus, contribution by other yet unidentified protease(s) to TRAP processing must also be implicated. In a recent study [242], proteolytic processing of TRAP over-expressed in the fibroblast-like CHO-K1 cell line was shown to be independent on cysteine proteinases, further corroborating the importance of proteases other than cysteine proteinases for the proteolytic processing of TRAP. The identification of such protease(s) and the subcellular localization of this processing event should help to clarify the coupling between proteolytic processing and intracellular transport of the TRAP protein.

Thus, the increase in total TRAP activity was mainly due to increased cellular content of monomeric TRAP. The increase in monomeric TRAP expression was more pronounced in osteoclasts of the distal compared to the proximal part of the metaphyseal trabecular bone, suggesting that proteolytic processing of TRAP is partially or completely dependent on cathepsin K *in vivo* depending on the precise localization of osteoclasts within the trabecular bone.

Moreover, examination of bone sections by transmission electron microscopy showed incompletely developed ruffled borders, denoted intermediate zones (ImZ), in a majority of osteoclasts from *Ctsk*<sup>-/-</sup> mice, suggesting a role for matrix modification by cathepsin K for proper polarization.

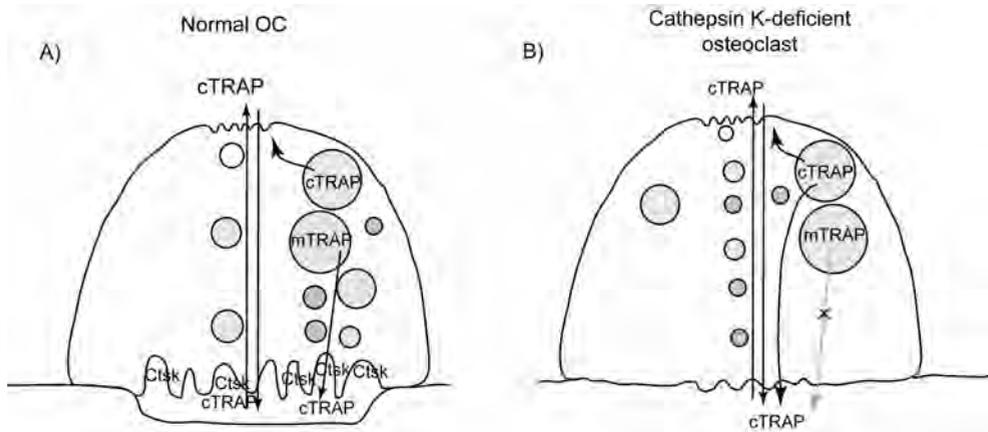
Additionally, TRAP was secreted into the intermediate zone as the processed form in osteoclasts of *Ctsk*<sup>-/-</sup> mice, unlike in osteoclasts from wild-type mice, which secreted TRAP to the resorption lacuna as the monomeric form, suggesting presence of cathepsin K-dependent as well as -independent mechanisms for TRAP secretion.

The results demonstrate that cathepsin K is not only involved in proteolytic processing but also affect the intracellular trafficking of TRAP, particularly in osteoclasts of the distal metaphysis.

In TRAP-deficient-mice (**paper III**), with a complete lack of TRAP protein expression, there was a notable accumulation of cytoplasmic vesicles indicating a disturbance of vesicular transport in osteoclasts. The concentration of cathepsin K in the ruffled borders of these TRAP  $-/-$  osteoclasts was however normal, indicating that the secretory pathway is not affected. On the other hand, in Ctsk  $-/-$  mice, with an accumulation of monomeric, enzymatically inactive TRAP in osteoclasts, the secretory pathway appeared to be altered with a shift in the secretion from the monomeric to the processed forms (Fig. 13). The results from **paper III and IV** collectively indicate a role for the cathepsin K-TRAP axis in the regulation of intracellular vesicular transport in osteoclasts. The precise mechanisms for how this regulation is exerted at the molecular level remains an interesting challenge for future studies.

In recent years, the activity of cleaved TRAP i.e. TRAP 5b in serum has become a popular marker to monitor bone resorptive activity associated with osteoclast numbers [243]. The studies in **paper IV** using Heparin affinity chromatography to separate different cleaved forms of TRAP demonstrate that only part of the serum activity of TRAP, representing a specific cleavage product, is due to the action of cathepsin K. This finding raises the interesting possibility that the cleavage profile of TRAP in serum could be related to the extent and/or mechanism of bone matrix degradation more accurately reflecting resorptive activity of osteoclasts compared to assays measuring total cleaved TRAP activity.

Importantly, this study highlights functional differences between bone –resorbing clasts within the trabecular metaphyseal bone, suggesting potentially important differences in the regulation of differentiation and activation depending on the precise anatomical localization of the clast population. A functional difference between osteoclasts in calvarial and long bones with regard to quantitative differences in cathepsin vs MMP-dependent collagen degradation has been established [120], and the data presented in this study extend the concept of osteoclast heterogeneity also to osteoclast populations within a trabecular bone compartment. These findings raise important questions as to the mechanisms underlying such phenotypic differences, e.g. whether the osteoclasts at different sites derive from different progenitor cells, or represent different stages of differentiation or are regulated by local factors present in the microenvironment of the cell e.g. the bone matrix or the stroma.



**Figure 13. Hypothetical model of vesicular transport of different TRAP forms in normal and Ctsk-deficient osteoclasts. mTRAP - monomeric TRAP; cTRAP – cleaved TRAP**

In normal osteoclasts, mTRAP is secreted to the ruffled border, where Ctsk removes a loop domain in mTRAP forming cTRAP [141]. This cTRAP associates with the matrix and is eventually transcytosed along with matrix remnants to the functional secretory domain on the basolateral side [113]. In addition, some cleaved TRAP is directed from intracellular sites to the functional secretory domain [156]. In Ctsk-deficient osteoclasts, the secretion of mTRAP to the ruffled border is blocked, and mTRAP accumulates in the cytoplasm. Intracellularly cleaved TRAP is secreted to the ruffled border, possibly through the transcytotic pathway, partially restoring the content of active TRAP in the resorption lacuna.

# CONCLUSIONS

From the results presented in this thesis the following major conclusions were drawn:

- Mineralization of bone matrix is required for osteoclast polarization but not for secretion of proteolytic enzymes.
- Matrix mineral is protective for and limits collagen degradation.
- The validity of osteoclast polarization as an ultrastructural morphological indicator of osteoclast resorptive activity may be restricted to bone with normal mineralization.
- Genes encoding mineralization-promoting proteins are activated in osteoblasts of hypophosphatemic rachitic mice, possibly as a compensatory mechanism to favour mineralization.
- Secretion of cathepsin K and TRAP to the resorption lacuna are differently regulated in polarized osteoclasts, suggesting a role for secreted TRAP in the later stages of the resorption sequence.
- TRAP deficiency affects osteoclast morphology and growth plate maturation
- Cathepsin K and TRAP are involved in regulation of osteoclast membrane traffic.
- Proteolytic processing of TRAP is mediated by cathepsin K in vivo.
- Osteoclasts at different locations in trabecular bone are functionally heterogeneous with regard to proteolytic processing of TRAP.

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