From Department of Laboratory Medicine Division of Pathology
Karolinska Institutet, Stockholm, Sweden

ROLE OF TARTRATE-RESISTANT ACID PHOSPHATASE IN BONE REMODELING

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Stockholm 2019

Published by Karolinska Institutet.
Printed by Arkitektkopia AB, 2019

ISBN 978-91-7831-646-5

Role of tartrate-resistant acid phosphatase in bone remodeling

THESIS FOR DOCTORAL DEGREE (Ph.D.)

som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Månen Q9 ANA Futura, Alfred Nobels allé 8, våning 9, Karolinska University Hospital, Huddinge

Onsdagen den 18 December, 2019, kl 10:00

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In the loving memory of my grandmother and my father in law

POPULAR ABSTRACT

The skeleton is a dynamic organ that is constantly renewing itself to maintain its strength. Starting at birth and during childhood, bone grows and changes shape in order to adjust to physical requirements. As we get older, bone is lost, and this loss might lead to conditions such as osteoporosis. Bone growth/adjustment requires continually replacement of old bone by new one. This process is called bone remodeling, which is composed of bone resorption followed by bone formation. The bone resorbing cell, the osteoclast (OC), is one of the three main cells types in bone; the other are osteoblasts (OB), which form bone, and the osteocytes (OYs) which play a key role in translating mechanical signals into biological responses. When imbalances in the remodeling cycle occur, such as if the OCs work harder than they should, the result is bone loss, accompanied by a higher chance of fractures. Examples of excessive resorption are found in diseases like osteoporosis, rheumatoid arthritis, glucocorticoids treatments and some cancers.

Tartrate-resistant acid phosphatase (TRAP) is an enzyme existing as two isoforms, TRAP 5a and TRAP 5b. OCs produce vast amounts of TRAP isoforms, however, little is known about the role of these isoforms in bone remodeling. Therefore, the aim of this thesis was to investigate the role of TRAP isoforms in bone remodeling. For this, we developed an ELISA that allowed us to measure the concertation of both TRAP isoforms in different samples. We found that OCs can secrete both isoforms and that they correlate to resorption markers. This is a new discovery, as it has never been reported before that OCs secrete TRAP 5a, which is a marker for chronic inflammation. We also studied the expression of TRAP isoforms in synovial fluid from rheumatic diseases. TRAP can activate other proteins by removing phosphate groups. Osteopontin plays a role in inflammatory diseases and TRAP is able to modify its configuration to make it more active. We showed that decreased levels of TRAP 5b could induce inflammation in rheumatoid arthritis. We theorized that the lower formation of OCs in an inflammatory environment, decreases the OC-mediated conversion of TRAP 5a to TRAP, leading to an inflammatory response and finally to the disease. We finally studied the biological role of TRAP by using an inhibitor of the enzymatic activity of TRAP 5b, and found that OC formation was mostly impaired and the expression of OC markers was downregulated. However, the OC that survived, were still active and able to resorb. Overall, TRAP seems to play an important role in bone diseases, and TRAP malfunction seems to be a direct cause of bone abnormalities. We do not know yet which mechanisms TRAP controls inside the OC, but our results suggest that OC differentiation is affected by TRAP inhibition and that less cells were able to fuse in presence of the inhibitor. This potentially opens the door for new treatments to treat bone loss.

ABSTRACT

Tartrate-resistant acid phosphatase is a metalloenzyme that exists as two isoforms: the monomeric TRAP 5a and the proteolytically cleaved TRAP 5b, responsible for phosphatase activity, which is highly expressed in osteoclasts (OCs). TRAP 5b has been used as a serum marker of bone resorption, as it correlates with the absolute number of OCs and with resorption markers such as CTX-I. Despite being used as biomarker for bone metabolic diseases, little is known about the role of TRAP isoforms in OCs and thus bone remodeling (the process of bone degradation by osteoclasts, and bone formation by osteoblasts). Therefore, this thesis aimed to investigate the role of TRAP isoforms in bone remodeling.

To enable the investigation of TRAP 5a and 5b we (1) developed a sandwich TRAP 5a/5b ELISA for the quantification of human TRAP isoforms. This ELISA was then used for (2) evaluating the expression and secretion pattern of TRAP 5a and 5b in healthy individuals and during OC differentiation. Additionally, we used the ELISA to (3) investigated if TRAP protein levels correlate to osteoarthritis (OA) or rheumatoid arthritis (RA). Here we correlated the phosphorylation status of the known TRAP *in vivo* substrate, osteopontin, to the TRAP isoforms. (4) Using a competitive inhibitor for TRAP 5b, we studied the role of TRAP in OCs differentiation. Lastly, we (5) developed a high throughput system to identify a subclone in a murine cell line that is a more homogeneous and stable OC precursors that could be used as a screening tool for OC biology studies.

A double TRAP 5a/5b sandwich ELISA was developed and designed as a two-step process. Using the ELISA, we showed that *in vitro* cultures of OCs secrete not only TRAP 5b but also TRAP 5a and that both isoforms were present intracellular establishing that 5b can also be formed intracellular in OCs. Correlation between TRAP 5a and 5b indicated a dependence between TRAP 5a and formation of 5b. There was a positive correlation in both serum from healthy men, and media from *in vitro* OC cultures of not only 5b but also TRAP 5a with CTX-I further suggesting that TRAP 5a also originates partly from OCs. Measurement of TRAP 5a and 5b in synovial fluid from OA and RA patients revealed a correlation between low TRAP 5b/ TRAP 5a ratio and phosphorylated osteopontin. This suggested that synovial fluid from RA patients contained an insufficient amount of TRAP 5b increasing levels of phosphorylated OPN leading to a higher OC activation and bone destruction. Inhibition of TRAP 5b using the competitive inhibitor, 5-phenylnicotinic acid, decreased the number of OCs formed and the expression of several OC markers. However, some OCs were able to fuse and resorb bone.

In this thesis we show that measurement of TRAP isoforms protein is an important tool in research and possibly also in diagnostic to understand the biological implications of TRAP 5a and 5b in OCs, which may lead to therapeutic targeting of certain isoforms for inflammatory and metabolic bone diseases. We further show that TRAP is involved in the bone remodeling process in OCs and defects in TRAP may cause alterations in OCs function and differentiation.

LIST OF SCIENTIFIC PAPERS

I. **Laia Mira-Pascual**#, Christina Patlaka#, Suchita Desai, Staffan Paulie, Tuomas Näreoja, Pernilla Lång, Göran Andersson. A novel sandwich ELISA for Tartrate-Resistant Acid Phosphatase 5a and 5b protein reveals that both isoforms are secreted by differentiating osteoclasts and correlate to the Type I collagen degradation marker CTX-I in vivo and in vitro. Calcif Tissue Int (2019). [Epub ahead of print]. Doi: 10.1007/s00223-019-00618-w.

II. Jani Luukkonen, **Laia Mira-Pascual**, Christina Patlaka, Pernilla Lång, Sanna Turunen, Jussi Halleen, Tomi Nousiainen, Maarit Valkealahti, Juha Tuukkanen, Göran Andersson, Petri Lehenkari. Increased amount of phosphorylated proinflammatory osteopontin in rheumatoid arthritis synovia is associated to decreased tartrate-resistant acid phosphatase 5B/5A ratio. PLos One (2016). 12(8):e0182904. Doi: 10.1371/journal.pone.0182904.

III. **Laia Mira-Pascual**, Anh N Tran, Göran Andersson, Tuomas Näreoja, Pernilla Lång. A subclone of RAW264.7 forms osteoclast-like cells capable of bone resorption faster than RAW264.7 through increased de novo expression and nuclear translocation of NFATc1. Submitted 2019.

IV. **Laia Mira-Pascual**, Suchita Desai, Anh N. Tran, Christina Patlaka, Pernilla Lång, Angela Casini, Barbro Melgert, Ulf Lerner, Petra Henning, Tuomas Näreoja, Göran Andersson. Tartrate-resistant acid phosphatase activity promotes RANKL-stimulated osteoclast differentiation in vitro. Manuscript.

equal contribution

Related article published by the defendant, not included in the thesis:

Christina Patlaka, **Laia Mira-Pascual**, Staffan Paulie, Anni-Frid Henriksson, Peter Arner, Pernilla Lång, Göran Andersson. The adipokine tartrate-resistant acid phosphatase 5a in serum correlates to adipose tissue expansion in obesity. Biomarkers 22, 764774 (2017).

CONTENTS

ABBREVIATIONS

5PNA	5-phenylnicotinic acid
AP-1	Activator protein-1
ALP	Alcaline phosphatase
BMU	Basic multicellular unit
BMD	Bone mass density
BSP	Bone sialoprotein
CtsK	CathepsinK
CTX-I	C-terminal telopeptide of type I collagen
ECM	Extracellular matrix
Fe^{2+}	Ferrous
Fe^{3+}	Ferric
FBGC	Foreign body giant cell
FSD	Fuctional secretory domain
hPBMCs	Human peripheral blood monocytes
IIAA	Immuno-immobilization activity assay
PPi	Inorganic pyrophosphate
IL	Interleukin
M-CSF	macrophage
MMP-9	Metalloproteinase-9
MITF	Micropthalmia transcription factor
mAb	Monoclonal antibody
NFATc1	nuclear factor of activated T-cells cytoplasmic 1
OA	Osteoarthritis
OB	Osteoblast
OC	Osteoclast
OY	Osteocyte
OPN	Osteopontin
OPG	Osteoprotegerin
PTH	Parathyroid hormone
RANKL	receptor activator of NF-kB ligand
RANK	receptor activator of NF-kB
RA	Rheumatoid arthritis
RGD	Arg-Gly-Asp peptide

RF	Ruffled border
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
SIBLING	Small integrin-binding ligand, N-linked glycoprotein
SZ	Sealing Zone
SPENCD	Spondyloenchondrodysplasia
SLE	Systemic lupus erythematosus
TRAP	Tartrate-resistant acid phosphatase 5
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TRIP-1	TGFβR-interacting protein -1

1 INTRODUCTION

Bone is a complex, vascularized and highly dynamic tissue that together with cartilage forms the skeletal system. It has to be strong and stiff to provide attachment points to muscles when they propel and support the whole body. However, it also needs to be flexible enough to absorb the loading energy from mechanical forces[1–4].

The skeletal system not only provides support and protection. The skeletal system not only provides support and protection of our internal organs, but it is in the red bone marrow within the bones of the skeleton, that red blood cells are formed. Bone is also considered as a metabolic organ because it serves as a calcium and phosphorus reserve to maintain normal physiological levels of both ions[5].

In general, bone *modeling* is the mechanism that alters and gives the shape of bones throughout life, whereas bone *remodeling* is the constant turnover that maintains and repair the bone. Bone turnover is high during the first year of life and later, approximately 10% of the bone is renewed every year with a complete renewal every 10 years[6].

1.1 Anatomy of bone

Bone tissue consists of organic and inorganic matrix components as well as four main bone cell types; bone-resorbing cells (osteoclasts; OCs), bone-forming cells (osteoblast: OBs) and two osteoblast-derived cells, called osteocytes (OYs) and bone lining cells. Additionally, bone has substantial extracellular matrix (ECM) consisting of mineral and protein components.

Morphologically, bone contain two different kinds of osseous tissue containing the same cells and matrix composition that can be distinguished based on porosity: **Cortical bone** is highly compact in structure while **trabecular bone** is porous. Cortical bone forms the condensed and hard exterior, and represents 80% of the total bone mass. It is full of microscopic tubes that contain the bone's blood vessels and nerves, called Haversian canals. Trabecular bone represents 20% of the total bone mass, contained in the internal spaces. It represents 90% of the bone area which is in contact with the bone marrow and vessels, this make trabecular bone more susceptible to bone remodeling[7] (**Figure 1**).

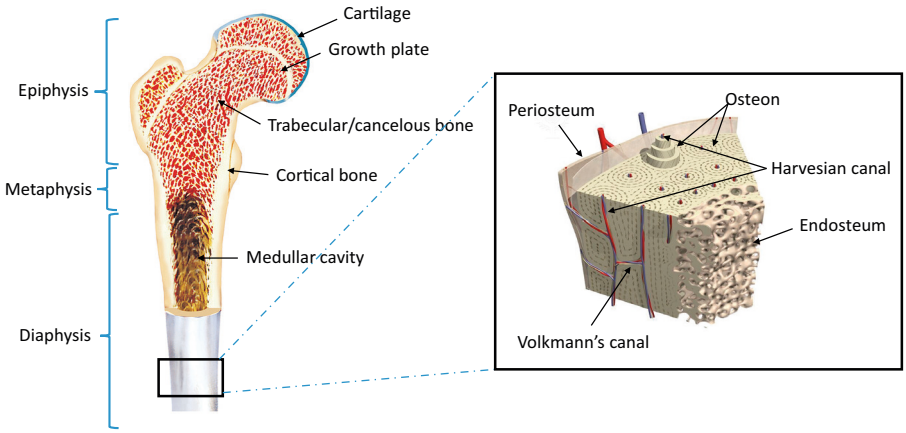

Figure 1. Anatomy of long bone. Structural organization of the proximal end of a femoral long bone. Enlarged diagram of compact bone from a transverse section of a long bone's cortex, where the osteon is shown with all the layers, and the canaliculi formed by the osteocytes. In the center is the Haversian canal where the blood vessels is located. Adapted with permission of from Encyclopædia Britannica, Britannica ImageQuest: structure of long bone, and compact bone, artwork; 25 May 2016. http://quest.eb.com. Accessed 28 Oct 2019.

Skeletal development

During embryonic development in higher vertebrates (birds and mammals), the first sign of bone development is the formation of mesenchymal condensations, in which mesenchymal progenitors aggregate at the future skeleton location. Skeletal formation occurs by two different means of developmental processes, either ***intramembranous*** or ***endochondral*** ossification. On intramembranous ossification, the formation of new bone involves the direct differentiation of mesenchymal cells into OB to form membranous bone. During endochondral ossification the migration and condensations of the mesenchymal cells gives rise to chondrocytes to form a cartilage model that will serve as a template to be replaced with mineralized bone through remodeling. While long bones i.e. tibia, femur are formed by this two processes, flat bones such as *calvaria* or *scapula* are formed by intramembranous ossification[8–10].

1.2 Bone cells

Osteoclasts – bone resorbing cells

OCs are an unique type of exocrine cell that dissolve the inorganic hydroxyapatite mineral matrix and enzymatically degrades the organic matrix i.e. collagen type 1 (CTX-I)[11]. OC are multinucleated cells derived from the hematopoietic myeloid lineage that arise from fusion of monocytes[12]. These mononuclear precursors can either migrate directly from the bone marrow to the bone surface or be first

attracted to the peripheral blood circulation and then recruited to the bone surface by different factors e.g. sphingosine-1 phosphate[13], where they mature into bone resorbing OCs[14,15]. Their differentiation is principally regulated by the regulator system consisting of macrophage colony stimulating factor (M-CSF, encoded by CFS1), receptor activator of NF-kB ligand (RANKL; encoded by Tnfsf11), its membrane receptor activator of NF-kB (RANK; encoded by Tnfrsf11a) and the soluble factor osteoprotegerin (OPG; encoded by Tnfrsf11b)[16].

Osteoblast – bone formation cells

On the other hand, OBs originate from the mesenchymal lineage and their differentiation is regulated by a plethora of hormones and cytokines e.g. parathyroid hormone (PTH), estrogen, glucocorticoids and vitamin D[17]. The stem cell commitment towards the OB lineage involves the expression of specific genes, including the master gene of osteoblastic regulation: Runt-related transcription factor 2 (Runx2)[18].

Mature OBs produce an unmineralized organic bone matrix (osteoid) composed of collagen type 1, osteopontin (OPN) and sialoproteins which aid to its mineralization[11]. OB functions include (1) regulation of bone-resorbing activity by a wide range of compounds e.g. prostaglandin E_2, interleukin-1(IL-1), IL-6 and RANKL[16,19,20] and (2) osteoclastogenesis through the RANKL/RANK/OPG pathway[21–23] among others pathways e.g. Notch signaling[24,25].

Bone lining cells/stromal cells

Bone lining cells are inactive OB, characterized by its flattened cell morphology with fewer organelles, and cover the bone surfaces not going through bone formation or remodeling. They reside in the outer part of the trabecular bone (endosteum) and the outer part of the cortical bone (periosteum), like a protective layer, thus preventing the homing of other cells to the bone surface. These lining cells are thought to have a role in the recruitment of OC[26] and in coupling OC and OB activity during the bone remodeling cycle by removing the protruding collagen from the bone surface and allowing the OB to form new bone[27].

Osteocytes – bone mechanoreceptors

Most past studies agree that OYs are terminally differentiated OBs that were trapped within the resorption lacuna after matrix deposition but recently this is debated as it has been shown that OYs have the ability to regain an OB phenotype[6]. OY have been described as the neurons of the skeleton because of their characteristic shape with canaliculi (tiny channels) communicating the bone cells and the vascular system with the bone surface[28–30]. OY death by micro-damage has been suggested to translate the mechanical loads forces into biological signals that regulate bone remodeling[31,32].

1.3 Extracellular bone matrix (ECM)

Bone matrix is composed of an organic based framework and an inorganic mineral phase. The major organic component is collagen type I fibrils (about 90% of the total protein mass), and it is upon these collagens fibrils that the inorganic hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals are deposited and confer strength and stiffness to the bone. The remaining 10% of the organic phase contains a relatively small amount of some minor types of collagen III and IV, fibronectin and non-collagenous proteins, including proteoglycans, glycosylated proteins such as small integrin-binding ligand (SIBLING), osteocalcin, and lipids and the bone cells[33]. An important subgroup of non-collagenous proteins is the RGD-containing proteins such as bone sialoprotein (BSP), OPN, osteocalcin, thrombospondin and vitronectin. Integrins that recognize the RGD sequence mediate cell-matrix contact[34,35]. Most of non-collagenous proteins are synthesized by the OB but the liver synthesizes approximately a quarter of them which are absorbed directly through the ECM. Osteocalcin is the more abundant non-collagenous protein produced by the OB (approx. 1%) and may play a role regulating bone formation as it was suggested by the increased bone mass observed in the osteocalcin knockout mice[36].

Osteopontin

OPN is expressed not only in the bone matrix but in a variety of soft tissues e.g. vessels and immune cells[37,38] e.g. T cells. OPN is a highly phosphorylated glycoprotein believed to be secreted by OCs and deposited into the resorption lacunae during bone resorption[39]. OPN belongs to the SIBLING family and is characterized by its flexible structure that enables OPN to interact with other proteins e.g. collagens and hydroxyapatite crystals[40]. When dephosphorylated – by for e.g. Tartrate-resistant acid phosphatase 5b (TRAP 5b) or alkaline phosphatase (ALP), (see sections 3.3 and 3.2.4). OPN-facilitates OCs adhesion and initiates osteoid mineralization[41]. In its phosphorylated form it inhibits hydroxyapatite crystal formation and growth[42].

The main role of OPN during inflammation is to trigger myeloid and lymphoid cells to secrete cytokines to drive the inflammatory/immune response[38].

1.4 The bone remodeling cycle

To maintain a healthy skeleton with optimal mechanical integrity, bone is constantly remodeled throughout life. The bone remodeling cycle is necessary for (1) fracture healing and (2) adaptation to mechanical forces by modifying the structure of bone and repairing micro-damage[26,43]. (3) It also serve to maintain calcium homeostasis[44]. During the remodeling cycle, the integrity of the skeleton is maintained by the coordinated formation and resorption of bone, enabling to replace the foci and damaged bone by new bone, thus helping to keep bones healthy.

The coupled activity of OBs and OCs is mainly determine by bone remodeling. Coupling refers to the coordinated actions of OCs and OBs during remodelling. Damage or stress applied to the bone can be sensed by the OYs and activate the bone remodeling cycle. These actions are performed in clusters arranged within temporary anatomical structures known as the ‘’Basic Multicellular Unit’’ (BMU)[45,46]. While each BMU occurs in a determined space and within a specific time it can occur at many times and places asynchronously through the whole skeleton.

The way the remodeling happens depends on the type of bone that is being remodeled, even though the distinction is more morphological than physiological as in both cases the cellular events follow the same sequence. The remodeling process can be further classified in two types: The Harversian remodeling within the cortical bone and the endosteal remodeling along the trabecular bone surface (**Figure 2**). In cortical bone the OCs, dig a tunnel, followed by the OBs that will fill with osteoid. In trabecular (also called cancellous bone) the BMU is similar, but it moves across the trabecular surface, where the OCs dig a trench rather than a tunnel[47,48]. In trabecular bone the capillaries or vessels are always close to the remodeling unit [49,45]. Both osteons and the new trabecular bone are aligned towards the dominant load direction.

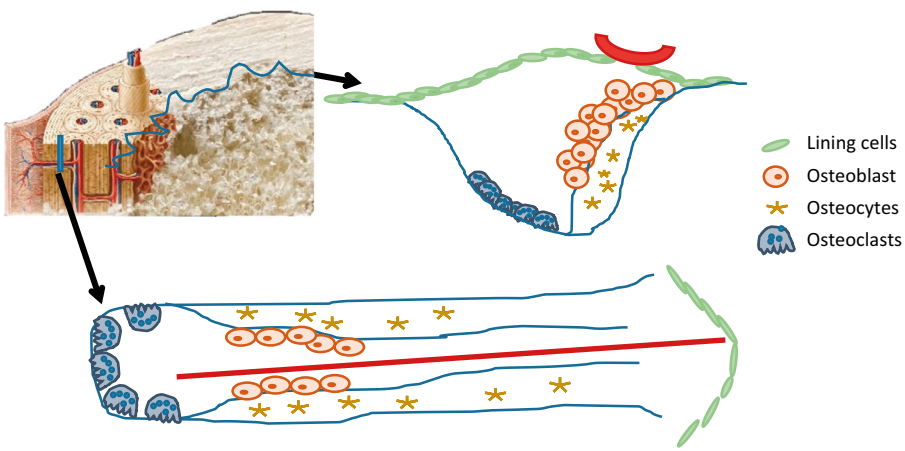

Figure 2. Cortical and trabecular remodeling on a long bone. Remodeling requires removal of bone by the OC and production of bone by the OB, thus there is coupling in the activity of both cells. In the remodeling process OC mobilize material, which leads to the recruitment of OB precursors to the bone surface that has been resorbed. The OB precursors differentiate into mature OB, and in normal circumstances replace the old bone that has been resorbed by the OC. Modified from: *Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit* and from *Assessment of bone vascularization and its role in bone remodeling*[50,51].

The traditional bone remodeling dogma describes five sequential phases of bone remodeling that occur in an active BMU on trabecular bone: (1) initiation/activation of OCs formation, (2) resorption mediated by OCs, (3) reversal period, (4) matrix formation by OB, and (5) mineralization/termination. Communication pathways exist among each of the four cell types mentioned previously[45].

The reversal phase of the remodeling is the least well understood. After OC have resorbed bone, the surface (Howship's lacunae) remains covered with undigested demineralized collagen[27]. Coupling of bone formation and bone resorption requires the recruitment of osteoproteginors, these osteoprogenitors are also named "reversal cells", and probably originate from the OB lineage[48]. The reversal cells' job is to remove the leftovers of collagen and to prepare the bone surface for the next phase, bone formation. The way they achieve this goal is still very controversial but is probably done by producing or receiving coupling signals allowing bone formation within the BMU[27,45].

Signaling in bone metabolism

Bone remodeling is a tightly regulated process modulated through the interaction and communication of OBs, OYs and OCs. Bone diseases are characterized by an imbalance of these processes, for example during inflammation or in several bone diseases e.g. osteoporosis, where bone resorption exceeds the formation or bone formation exceeds resorption e.g. osteomalacia, osteopetrosis. (**Figure 3**)

Bone metabolism is regulated by several signaling pathways and soluble factors secreted by different organs. Bone formation is regulated by the WNT-β-catenin signaling pathway[52–54]. This pathway enhances the differentiation of mesenchymal cells into OB[55]. There are antagonists that prevent the differentiation of OBs, and prevent bone formation such as sclerostin[56] and dickkopf-related protein 1[57]. Excess of vitamin A can also inhibit OB differentiation[58,59].

The OPG-RANK-RANKL pathway is required to regulate bone formation and resorption[60]. RANKL is secreted by both OBs and OYs, but OYs have been identified as the main regulators of bone remodeling[61]. When RANKL binds to its receptor, RANK on the osteoclast stimulates bone resorption. OB produce OPG which is a RANKL decoy receptor as it impedes RANK-RANKL interaction by binding RANKL. Recently, has been identified the RANKL reverse signaling. Here, OCs secrete vesicular RANK that can then bind on the RANKL exposed on the OBs surface [62]. This pathway is detailed in reviewed in section 2.1.

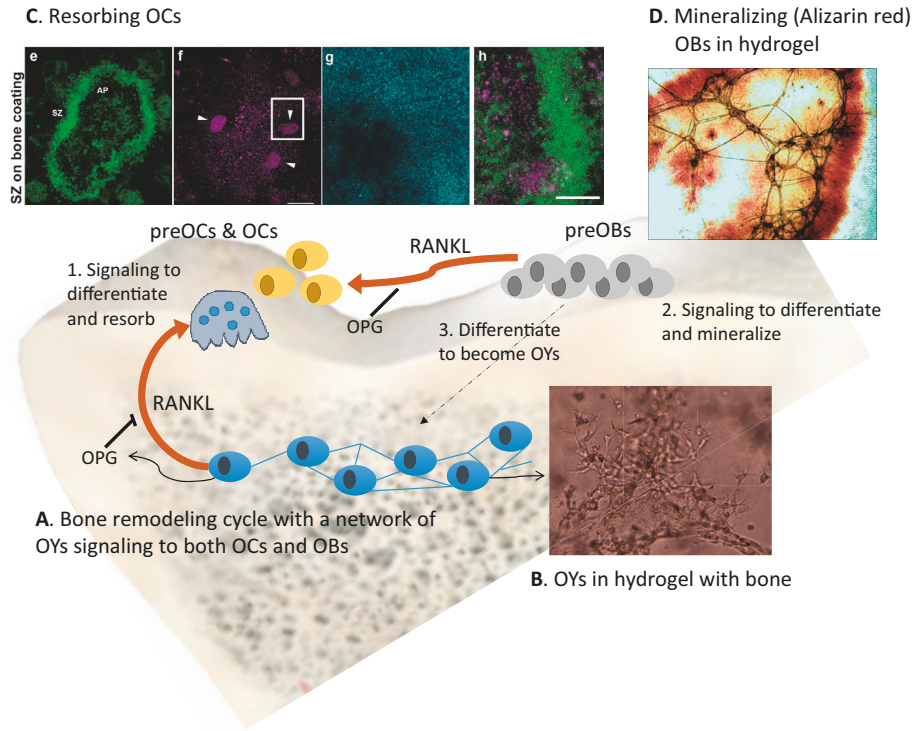

Figure 3. Bone remodeling cycle with a network of OYs signaling to both OCs and OBs. 1) Initiation/activation for OCs to differentiate and resorb **2**) the OYs network secrete RANKL after sensing changes, mechanical strain, microdamage, bone resorption done by an OC, **3**) Bone deposition and mineralization by the OB. *By kind permission of my supervisor Tuomas Näreoja, Karolinska Institutet.*

1.5 Coupling of bone formation and resoption

Bone deposition and bone resorption are ongoing dynamic processes. Several coupling signal have been proposed, some produced by OCs[63] including TRAP 5a, sphingosine 1-phosphate and the cell-anchored EphB4-ephrin-B2[64]

There are different stimuli that contribute to coupling of the different components of the bone at the BMU such as (1) growth factors released from the matrix e.g. transforming growth factor β1 (TGFβ1). Recent evidences indicate that OC initiate remodeling by mobilizing and activating TGFβ1 that is stored in the bone matrix in a latent form. This mobilized growth factor targets mesenchymal stem cells (MSC), recruiting them to the bone surface where they differentiate into OBs, thereby completing bone remodeling at that particular site[47,65–67]. (2) soluble and membrane products from OCs or their precursors, e.g. semaphorin4D, that

inhibits bone formation during bone resorption[68,69] (3) signals from OYs e.g. slcerostin, inhibiting bone formation[70], (4) signals from the immune system e.g. tumor necrosis factor-α (TNFα) secreted by T cells enhances OC formation. In humans, defects in bone remodeling lead to a range of metabolic and genetic syndromes such as osteoporosis, Paget's disease and osteopetrosis[71].

2 OSTEOCLAST

General organization

OCs resorb bone via the specialized bone-resorbing membrane domain called, **the ruffled border (RB)** which is formed through trafficking and fusion of acid vesicles from the secretory pathway containing proton pumps and osteolytic enzymes[72] and is used as a hallmark of an active OC[73]. The formation of the RB is a critical event for OC function. RBs do not form in certain genetic cases of osteopetrosis, severely reducing OC resorption and resulting in an increase in bone mass[74]. The RB is located on the basolateral side of the OC facing the resorption lacuna and consists of irregular deep enfolding of the plasma membrane, covering the resorption area and mediating secretion of resorption factors and uptake of resorption products[75].

The ruffled border is surrounded by the **sealing-zone (SZ)** which consists of an actin ring delimitating the resorption lacuna and attaches the OC to the matrix via the $a_v\beta3$-integrin expressed in the SZ by the podosomes structures[76]. The attachment of the ring to the bone matrix is mediated by integrin receptors i.e. $\alpha v\beta3$, binding to RGD sequences in specific bone matrix proteins e.g. vitronectin and OPN[74]. The **clear zone**, refers to the cytoplasmic side of the SZ and is devoid of organelles, but rich in actin filaments[77]. Following the degradation of bone matrix, the RB uptakes the degraded products, that are then trafficked in vesicles through the cell via transcytosis, to the **functional secretory domain (FSD)** located on the basal membrane, to the circulation. The membrane facing the vascular capillaries is called the **basolateral domain**, and is presented with an asymmetrical distribution of ion transporters. A transcytosis route is stablished between the RB and the FSD of the basolateral membrane, that consent the released of the bone matrix degraded products to the blood stream (**Figure 4**)[73].

Polarization

OCs attach to the bone surface by binding its surface integrins to the bone proteins e.g. vitronectin and OPN. The attachment arrange the cytoskeletal configuration into a polarized, multinuclear, secretory cell with four major structures i.e. the RB, the SZ, basolateral domain, and FSD (**Figure 4**). This polarization enables the OC to become active and demarks the resorption lacuna which is formed by sealing off the area beneath the OC and forming the acidic microenvironment that is necessary to resorb bone[73,78–80].

Interestingly, it was shown in a rachitic fibroblast growth factor-23 transgenic mice that OC polarization is not required for degradation of bone matrix[81]. FGF23 is involved in phosphate homeostasis and vitamin D vitamin metabolism, and overexpression impairs, mineralization[82]. OCs could not attach properly to the bone surface and polarize but resorption was still on going through secretion of cathepsin K (CtsK) and metalloproteinase -9 (MMP9). However TRAP was not secreted.[81]

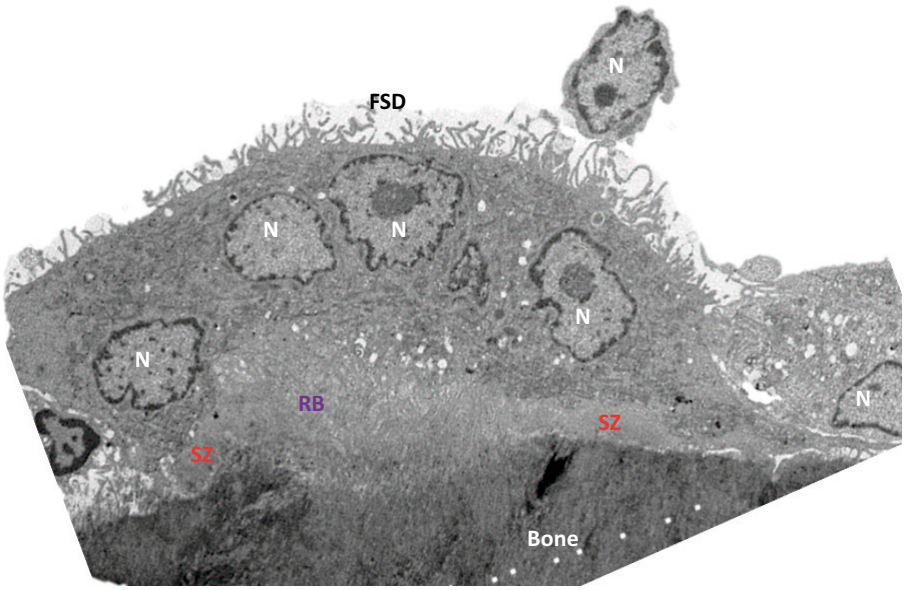

Figure 4. Cell organization in Osteoclasts. Transmission electron micrograph showing a rabbit osteoclast on dentine with 4 or 5 nuclei (N), sealing zone (SZ), ruffled border (RB) and the functional secretory domain (FSD. It can also notice that OC has abundant organelles and mitochondria. *By kind permission of E. McDermott and M.H. Helfrich, University of Aberdeen.*

Resorption

The detection of an initiating remodeling signal attracts the monocytes to the resorption site, which leads to their fusion to form an OC and attachment of the OC to the bone surface. This signal can take several forms e.g. direct mechanical strain on the bone that results in structural damage or also hormone action on bone cells (estrogen, PTH)[45]. The adhesion is mediated through αvβ3 vitronectin receptors.[83]

One of the features that clearly identify an active OC from any other cell type (macrophages, foreign body giant cells (FBGCs) is their ability to resorb bone and cartilage[84,85]. They resorb bone during bone growth and remodeling. Alterations on the balance of bone formation and resorption cause different bone related diseases. Bone resorption can only be performed by OCs due to their capability to degrade the bone matrix by (a) acidifying the mineral content and subsequent (b) degrading of the collagen content of the bone matrix[74,86].

Once OCs undergo polarization and the RB, the FSD and the SZ are formed, the OCs start the solubilization of minerals (mainly hydroxyapatite) by generating an acidic environment through pumping protons into the resorption pit. These protons are generated in the cytoplasm by the enzyme carbonic anhydrase II and the hydrogen ions are pumped via ATPase subunits, such as ATP6V03[87] and

ATP6V0d2[88,89], which are essential for OCs function into the resorption lacuna[90]. To maintain electroneutrality, chloride channel 7 in the RB transports Cl^- into the resorption lacuna[91]. The H^+-ATPase transports also enzymes such as CtsK, MMP9 and TRAP are secreted to the resorption lacuna to degrade the bone matrix. The collagen degradation (mainly type 1 collagen) is due to the activity of CtsK and metalloproteinases (MMPs). CtsK is a cysteine proteinase, that proteolytically degrades collagen into pieces at and acidic environment (pH ~5)[75,80]. Absence of CtsK[92] results in a mild osteosclerotic phenotype, suggesting that other enzymes might also contribute to the organic degradation during bone resorption. MMP9 is a MMPs expressed in several types of cells and implicated in the turnover of bone and cartilage matrix during bone remodeling. When CtsK knockout mice have a mild sclerotic phenotype the ablation of both CtsK and MMP9 causes severe osteopetrosis[93] (**Figure 5**).

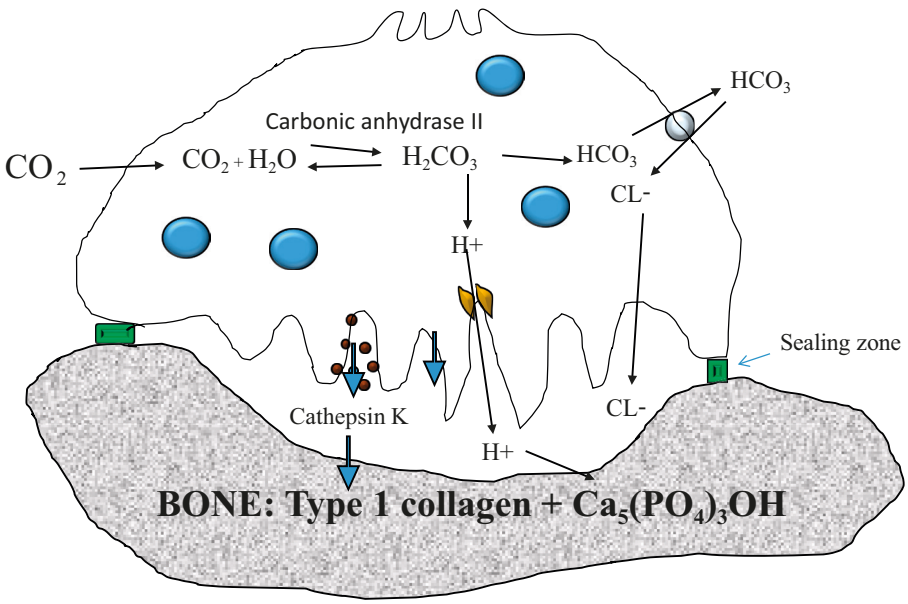

Figure 5. Resorption process. OCs resorb bone in sequence of steps: after attaching to the bone through the sealing zone it has to produce acid and it does that using the enzyme carbonic anhydrase II which catalyzes the reaction of carbon dioxide with water to generate carbonic acid, the carbonate component is eliminated from the cell, the protons are pumped across the ruffled border into the extracellular vacuole in the resorption space and chloride ions cross passively in order to maintain the electrochemical balance. The acid generated, dissolves the hydroxyapatite component of the bone. In order to break up the collagenous component of the bone, the OC uses CtsK which is secreted across the ruffled border. CtsK digests the collagen into smaller pieces, which are then endocytose into the OC and transported across the cell to the extracellular environment. The result of these actions is the formation of a resorption pit.

However, OC resorption does not only form resorption pits while immobilized, but yet another type of resorption have been proposed as it has been shown that OC can move across the bone surface while resorbing, forming trenches[80]. Since the SZ surrounds the resorption pit, it was shown that during trench-mood OCs move over the bone surface without disabling their resorption machinery. Trenches reflect long, fast and aggressive bone resorption *in vitro*[80].

It has recently been reported the existence of septins in OCs are expressed during differentiation[94]. Septins are known to support the cytoskeletal stability, vesicular transport, endocytosis and stabilizing cellular membranes[95]. The stabilization of septin filaments inhibits bone resorption and the conditional knockout mouse *sept9* leads to higher trabecular bone and lower femoral growth *in vivo*[94].

Vesicular transport

After synthesis in the endoplasmic reticulum (ER), the lysosomal enzymes are labelled with mannose-6-phospate residues, which are recognized by their receptor in the trans-Golgi network. They are either packaged into clathrin-coated vesicles and transported by transcytosis through the cell, to the FSD or kept for digestion via a series of processes including endocytosis, phagocytosis, and autophagy.

2.1 Osteoclastogenesis

OCs differentiation is mainly regulated by OBs and OYs through the RANK/RANKL/OPG axis. As mention in section 1.4., M-CSF and RANKL[43,76,96–99] are two essential cytokines involved in OC differentiation. Different signals enhance the production of these two key master regulators of OC differentiation e.g. low calcium levels enhance the production of calcineurin by the PTH hormones and thus enhance the levels of RANKL and M-CSF in order to restore the calcium levels[100].

The binding of M-CSF to its receptor, c-fms, provides signals for proliferation and survival that induce the expression of the receptor activator of nuclear factor kappa β (RANK) in OC precursors through the activation of ERK and Akt[101]. The importance of M-CSF in OCs differentiation is exemplified by a mice model for osteopetrosis (op/op), in which mice lack OCs and circulating monocytes due to a mutation in the coding region of the M-CSF gene. Addition of M-CSF to these mice restores OCs population and rescues the phenotype [12,102,103]

RANKL is the second key factor in OCs differentiation, which is expressed by OBs and OYs[96,97]. Mice lacking either RANKL[104] or RANK receptor[105] suffer from severe osteopetrosis and defective tooth eruption due to the complete lack of OCs. RANKL is a member of the TNF superfamily. The signaling activation RANK/RANKL requires the recruitment of adaptor molecules, several of them belong to

the TNF receptor associated factors (TRAFs), and while several of them can bind to RANK, TRAF6 is the one that preferentially binds to RANK in OCs precursors. Once TRAF6 has bound to RANK the recruitment of activated mitogen-activated protein kinases (MAPKs), and activator protein-1 (AP-1) and the transcription factor nuclear factor-κβ (NF-κβ)[106] is initiated.

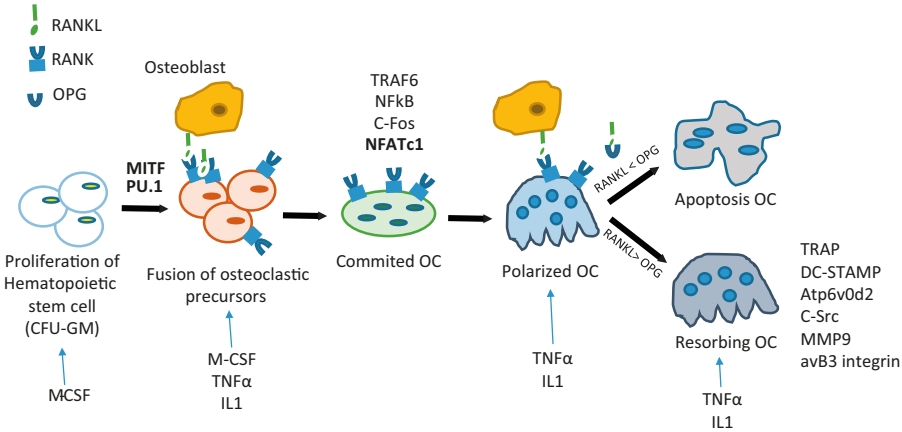

Figure 6. Overview of the different steps for osteoclastogenesis. Under physiological conditions the monocyte differention through bone resorbing osteoclasts is regulated by two cytokines, M-CSF and the RANKL. M-CSF is a survival factor that stimulates the ERK and the Akt pathway. Under the stimulation of others cytokines the differentiation can go in different directions, forming dendritic cells, M1 macrophages or M2 subtypes.

Activated NF-kB induces the activation of nuclear factor of activated T-cells cytoplasmic 1 (NFATc1 or NFAT2) which is a key regulator of OCs differentiation[107]. In addition to RANKL co-stimulatory signals, Ca^{2+} oscillation in osteoclast precursors activates phosphatase calcineurin which causes dephosphorylation of NFATc1 and translocation of c-Fos and NFATc1 to the nucleus, thus regulating the expression of OC-specific downstream target genes including TRAP, OC-associated immunoglobulin-like receptor (OSCAR), CtsK, calcitonin receptor, MMP9 and β3-integrin, leading to the development of mature osteoclasts[108–110]. NFATc1-knockout mice exhibits osteopetrosis due to the inhibition of OCs differentiation *in vivo* and *in vitro*[111].

Moreover, NFATc1 cooperates with other transcription factors such as the melanogenesis associated transcription factor (MITF) and PU.1, that regulate mononuclear pre-osteoclast fusion by inducing several genes involved in the fusion process, such as dendritic cell-specific transmembrane protein (DC-STAMP), osteoclast-stimulatory transmembrane protein (OC-STAMP)[112] and the d2 isoform of vacuolar (H+) ATPase (v-ATPase) Vo domain (Atp6v0d2). It was shown recently, that

knockout mice for DC-STAMP[113] or Atp6v0d2[114] develop osteopetrosis due to a defective fusion process during osteoclastogenesis, thus highlighting the importance of both genes in OCs fusion, to form bone-resorbing OCs.

In addition to OCs differentiation through RANK signaling, other pathways may also interact with NFATc1 signaling and stimulate or inhibit its translocation into the nucleus and subsequent induction of the target genes for cell fusion, OCs differentiation and function such as the triggering receptor expressed in myeloid cells-2 (TREM-2) and OSCAR –a collagen receptor, that actives OC on bone (**Figure 7**)[115–117]. Cell lacking NFATc1 were not able to differentiate to OCs, but also NFATc1 affects processes involved in resorption and cytoskeleton re-organization.

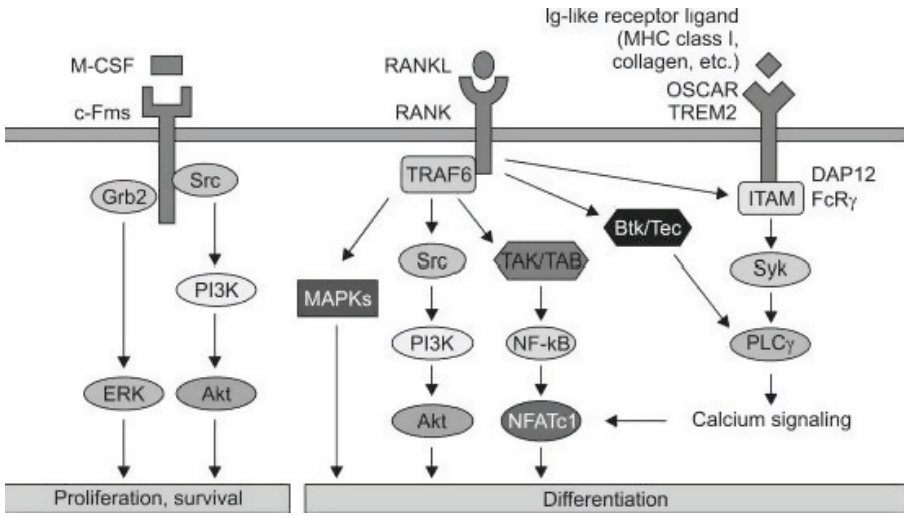

Figure 7. Osteoclasts signaling pathways. Schematic representation of M-CSF, RANKL and co-stimulatory signaling active during osteoclastogenesis. RANKL stimulates differentiation through the activation of different genes. Once NFATc1 is dephosphorylated and enters into the nucleus enables the activation of CtsK, chloride channel-7 (CLC-7), TRAP, DC-STAMP. Reprinted with permission of Jung Ha Kim and Nackcsung Kim. Signaling Pathways in Osteoclasts Differentiation[101]. Copyright © Chonnam Medical Journal, 2016 Jan; 52(1): 12–17

OBs also produce the endogenous RANKL inhibitor, OPG that is a soluble decoy RANK receptor for RANKL. Mice deficient of OPG show osteoporosis due to increased number of OCs[118]. It is worth noticing that similar effects can be obtain by using a synthetic antibody against RANKL, called Denosumab, which is being used clinically to prevent bone loss[9]. Thus, the ratio between RANKL to OPG is critical for the skeleton. When the amount of RANKL exceeds the amount of OPG, bone lose will be increased and conversely when OPG exceeds RANKL, bone lost will be prevented. Any imbalance in bone deposition and resorption results in

many metabolic bone diseases. Defects in the removal of mineralized bone or in bone resorption result in a deficient deposition of bone that lead to osteopetrosis, resulting in dense but brittle bones. On the other hand, if the resorption of bone exceeds the deposition of bone, osteopenia (low bone mass) can occur which may lead to osteoporosis reducing the strength of the skeleton and limiting the mineral available for metabolic functions.

2.2 Osteoclast heterogeneity

Although OCs have been long considered to be an homogenous population of bone resorbing cells, extensive research has demonstrated the existence of functional differences between OCs[34]. Bone-site specific OCs are phenotypically different e.g. in resorption machinery[9], drug intake and in response to local or systemic hormones e.g. PTH[119]. At least two explanations have been proposed to explain the presence of functionally heterogenetic OCs [9]; (I) It has been suggested that site-specific OC heterogeneity could be explained by the properties of the mineralized matrices (different microenvironment) they resorb and, (II) different monocytes subsets give rise to functional different OCs, for instance, under the influence of inflammatory cytokines.

OCs precursors

Three monocytes subsets can be distinguish based on the surface markers expression: the classical monocytes, with high expression of $CD14^{++}$ (LPS receptor), the non-classical monocytes with high expression of $CD16^{++}$(immunoglobulin Fc-domain receptor)[120], and the intermediate monocytes expressing high levels of $CD14^{+}$ and some $CD16^{+}$.

The non-classical monocytes, are predisposed to M2 activation, respond to larger antibody recognized targets, and are able to trigger an immune response with the consecutive release of cytokines recruiting more monocytes to the inflammatory site. These monocytes are more likely to respond to a combination of inflammatory and osteoclastogenic stimuli and differentiate into inflammatory OCs that are able to acidify external targets and promote inflammation[121,122]. Everts et al. suggested that classical monocytes are the main source of OCs, while the intermediate and non-classical monocytes contribute to inflammatory conditions, giving rise to FBGCs, immunological osteoclast-like cells related to the macrophage population[123–125].

Foreign Body Giant Cells (FBGCs)

FBGCs are a subpopulation of multinucleated giant cells[126] which develop through fusion of macrophages. This fusion of the monocytes/macrophages into FBGC is exclusively related to the presence of a foreign material[84,85]. Inflammatory conditions can cause a cross-react in signaling pathways that guide cell differentiation, differentiating them into either OC or FBGCs[84].

FBGCs and OCs share common characteristics such as TRAP expression and multinuclearity. However, there are several characteristics that differentiate a functional OC from an activated FBGCs. OCs secrete primarily TRAP 5b and express CtsK what allows them to resorb bone. On the other hand, FBGCs do not secrete TRAP 5b and are incapable of forming the RB or expressing CtsK. This makes them, even though they are able to acidify, fail to degrade collagen type I, thus failing to resorb bone[84]. Sprangers and Harkel performed a series of experiments in which they exposed the classical and non-classical monocytes to standard physiological conditions e.g. RANKL+MCSF and/or to inflammatory cytokines e.g. IL-4/IL-13[127]. IL-4 and IL-13 have been shown to participate in macrophage fusion *in vitro*[128–130] and IL-4 has also been involved in the activation of dendritic cell–specific transmembrane protein (DC-STAMP), a fusion related marker[131] essential for OC differentiation[132] and highly expressed in OCs and macrophages treated with IL-4[133]. IL-4 has also shown to promote fusion of monocytes in a RANKL-independent pathway[134]. Their results showed that the non-classical monocytes treated with inflammatory cytokines were larger, with more nuclei, and exhibited acting rings but were not able to resorb bone and were consequently classified as a macrophage subpopulation of FBGCs[84,135].

Bone-site specific OCs

Everts et al., (2008) studied the protein composition between flat and long bones and suggested that the different bone origin could explain the functional differences observed in OCs[119,136]. Beertsen et al., (2009) showed that MMPs participate in resorption of calvarial bone but not in long bone resorption while CtsK and CstL participate in resorption at both bone sites, even though their activity levels are higher in long bone located OCs than in calvarial OCs[119]. Saftig et al, (1998) reported that CtsK knock-out mice have an osteopetrotic phenotype in long bones while their flat bones are normal, also indicating that different bone-site specific OCs might be have different functions due to differences in the expression of CtsK during resorption, leading to resorption defects[92,137]. Everts et al., (2015) studied the relationship between bone site specific OBs, and OC heterogeneity. They found that a higher number of TRAP positive OCs arise from the calvaria OB, while the expression of OPG was higher in the long-bone OBs[138] possibly inhibiting differention of OCs.

2.3 Osteoclasts in inflammation

Apart from the two essential cytokines M-CSF and RANKL involved in physiological OC differentiation, some cytokines released during inflammation have been recognized to stimulate bone resorption in numerous rheumatic diseases. The most common types of arthritis are rheumatoid arthritis (RA) and osteoarthritis (OA). Inflammation is a common trait in both diseases; however, in RA the cause is autoimmunity while the cause is unknown in OA.

During synovitis cytokines released by infiltrated T cells e.g. TNFα and interleukin-17 (IL17), can up-regulate RANKL expression in OB and it is also possible that RANKL expressed by T-cells could increase OC differentiation[139] leading to an enhanced resorption and thus loss of bone mass. However, activated B cells are also part of the infiltrate, and are a major source of OPG, hence its contribution to RA osteoclastogenesis in unclear. When macrophages and monocytes infiltrate the synovia, they also secrete MMPs that can contribute to ECM degradation. Even though these cells can also secrete MMPs inhibitors, the balance seems to be favoring matrix degradation.

As mentioned above, negative regulation of OC differentiation is also controlled by the soluble decoy OPG, but under pathological situations cytokines e.g. IFNα, IL10 and IL4 have been shown to inhibit OC differentiation and activity [14].

Rheumatoid arthritis

RA is an autoimmune disease associated with chronic inflammation that results in local bone loss and joint deformations caused by increased OC resorption. In a joint affected by RA the synovium is swollen due to an infiltrate consisting of fibroblast-like and macrophage-like cells and several populations of T and B cells that secrete different pro-inflammatory cytokines able to increase bone resorption such as TNFα and interleukins like IL-1, IL-17 and IL-6, and thereby degrading cartilage and bone[140,141]. RA is an autoimmune disease and the presence of autoantibodies against common antigens expressed within and outside the joints – rheumatoid factorand anti-citrullinated peptides antibodies, is associated with a more severe RA phenotype.

The contribution of OCs in RA bone loss is demonstrated by the protective effects of bisphosphonates and the RANKL-neutralizing antibody denosumab, as well as elevated serum RANKL: OPG ratios correlating with radiological disease progression. Several mechanisms involved in the pathological OC activity have been elucidated. Antagonists against TNFα alpha, IL-6 and IL-1b have all been proved effective as anti-arthritis drugs particularly the TNFα inhibitors, with regard to reducing RA progression and bone erosion[142].

Osteoarthritis

OA starts as a cartilage disease that progressively results in a degenerative bone disease affecting joints. Little is known about the initiating events of OA, but repetitive use of the affected joints, genetic/epigenetic factors and particularly smoking may be implicated. Inflammation also contributes to the joint degradation and many inflammatory mediators in OA are similar to those in RA, with the exception of autoimmunity.

In a study done by Lövfall et al (2018)[143], OCs were set up in a culture model system where human OCs degrade cartilage from the knee joints of cows and they are subsequently treated with inhibitors for MMPs and CtsK. They measured different cartilage degradation biomarkers[143,144] and showed that the MMPs contributed strongly to the release of cartilage degradation markers while the contribution of CtsK was low.

2.4 Resorption inhibitors

Resorption inhibitors are drugs that inhibit OCs resorption or mineralization of the bone. They are used to treat bone loss in different diseases such as osteoporosis in postmenopausal women, glucocorticoid induced osteoporosis, Paget's disease of bone and and malignant hypercalcemia.

Bisphosphonates

Bisphosphonates are the primary pharmacological drug against OC mediated resorption, in order to prevent bone loss. However, implications of bisphosphates in pathological conditions such as osteonecrosis of the jaw has questioned its overuse in other skeletal disorders.

Structurally, bisphosphonates are inorganic pyrophosphate (PPi) derivatives with two phophonate groups ($PO(OH)_2$). It was shown in 1960s that PPi can inhibit mineralization through binding to the hydroxyapatite crystals. It was hypothesized that PPi regulates bone mineralization[145]. Bisphosphonates inhibit resorption by inducing apoptosis in OC[146]

Cathepsin K inhibitors: Odanacatib

As mentioned in section 2, CtsK is involved in collagen degradation, it is stored in lysosomes, and can regulate itself (proptide, glycosaminoglycans, oxidants) or be regulated by acidic conditions[147,148]. Defects in CtsK lead to pycnodysostosis, and autosomal recessive osteochondrodysplasia characterized by short stature, an increased bone mineral density and fractures. Inhibition of CtsK increases the bone mineral density in patients. Despite multiple efforts to develop an inhibitor for CtsK as an additional treatment for osteoporosis, concerns related to off-target effects have been considered, as CtsK is also found in a variety of tissues other than bone[149]. However, a double-blind phase III study of Odanacatib was approved to treat osteoporosis and the results were promising, Odanacatib was withdrawn from the regulatory approval after its use was linked to anincreased risk of cerebrovascular accidents. [150]

3 TARTRATE-RESISTANT ACID PHOSPHATASE

Tartrate-resistant acid phosphatase 5 (TRAP; TRAcP; ACP5, PAP) is a metalloenzyme and a member of the purple acid phosphatase family (EC 3.1.3.2) which was once considered purely a histochemical marker of osteoclasts (OCs) and hairy cell leukemia[151]. Besides OCs, the enzyme is also expressed in other bone cells such as osteoblasts and osteocytes[152,153] and on the surface of the bone matrix[153]. However, its role in bone remodeling is not well elucidated.

3.1 TRAP isoforms 5a and 5b

Acp5 gene and transcripts

The structure of TRAP gene is well conserved among species and contains five exons, with the initiation site located at the beginning of Exon 2[154,155]. There is one single TRAP gene (Acp5) giving rise to three transcripts in mice and seven in humans[156]. However, these transcripts give rise to the same protein but differ in their promotor and regulatory sequences[157]. Consequently, TRAP is likely regulated by different transcription factors in different cell types[158]. In mice, expression of transcript 1A is restricted to adult bone and spleen, transcript 1B is associated with expression in TRAP positive cells or tissues that are not from the myeloid lineage e.g. OBs[157]. Transcript 1C is the main transcript in OC and macrophages, and characteristic of TRAP expression in myeloid cells[157]. TRAP is translated as a single polypeptide and TRAP isoform 5a and 5b are a result of a post-translational modification.

Several transcription factors have been identified as master regulators of OCs development implicated in TRAP expression such as the microphthalmia Transcription Factor (MITF)[159] and PU.1[158] that recognizes specifically the sequence of the 1C promoter. Deletions on the PU.1 gene result in osteopetrosis due to impaired differentiation of myeloid cells. Transcriptional factor FoxM1 activating TRAP has also been linked to cancer metastases like in hepatocellular carcinoma[160]. NFATc1 has not been shown to interact directly with the Acp5 gene, but after NFATc1 expression increase in TRAP expression is found[161].

In murine RAW264.7 cells two regions are involved in RANKL-induced TRAP transcription, it was shown that the nuclear proteins that bind the promoter are upstream stimulatory factors (USF) 1 and 2. Mutations of these USF 1 and USF2 block RANKL-induced TRAP expression in RAW264.7 cells[162].

Protein structure

TRAP is found as two isoforms, TRAP 5a and TRAP 5b (**Table I**). TRAP 5a is translated as a single polypeptide ~35kDa and consists of 305 amino acids. TRAP 5b consists of two subunits that form a disulfide-linked heterodimer of 16kDa and 23kDa[163]. The sequence contains two potential N-glycosylation sites (Asn 97 and Asn 128) located in N-terminal fragment. Mutations in the these glycosylation sites reduced the substrate affinity for the catalytic site[164]. (**Figure 8**)

Table I. Properties of TRAP isoforms

	TRAP 5a	**TRAP 5b**
Sialic acid	Present	Not present
pH optimum	4.9-5.1	5.7-5.9
Phosphatase activity	Low	High
Structure	Intact ~35 KDa	Cleaved 16 Kda +25KDa
Expression	Secreted by OCs Secreted by Mφ	Secreted by OCs Intracellularly in Mφ

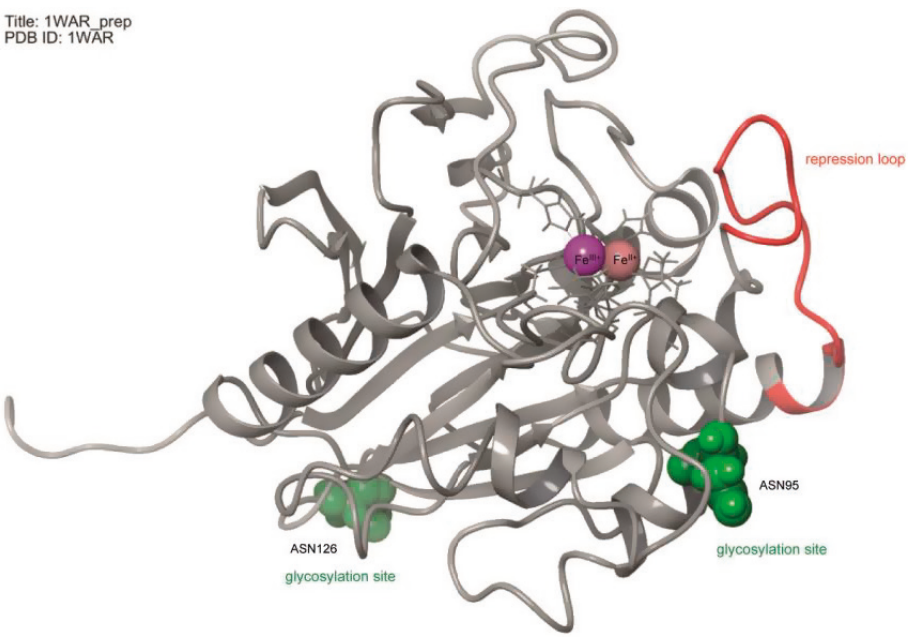

Figure 8. Recombinant human TRAP. Protein structure resolution 2.2 Å, PDB ID 1 WAR. Fe atoms are highlighted in the center, the repression loop is shown in orange and the two possible glycosylation sites are represented in green. *By kind permission of Anja Reithmeier, Karolinska Institutet.* Graphics by Biognos AB, Martin Frank, 2015.

Posttranslational processing of TRAP

In 1978 Lam et al., identified from a differential electrophoresis profile that TRAP exists in two isoforms in human serum. Two bands with different molecular weight were observed during electrophoresis and named as TRAP 5a and TRAP 5b[165], it was later suggested that this phenomenon was due to the presence of sialic acid residues on TRAP 5a[166]. However, it was later discovered that the difference between TRAP 5a and TRAP 5b was structural and was due to the presence of a flexible peptide loop of 20 amino acids that interacts with the active site of the enzyme and inhibits the reduction of the di-iron metal center preventing its enzymatic activity[167,168].

TRAP 5a can be subjected to post-translational proteolytic processing in the flexible peptide loop domain by different cysteine proteinases e.g. Ctsk [169,170]. The peptide loop has a sequence of 20 amino acids between aa 145-164, and it has been shown that depending on where the cut occurs, TRAP 5b will have different enzymatic activity[171]. (**Figure 9**)

It was first shown in 1993 by Orlando et al. that cleavage with trypsin of a five amino acids sequence in the loop region resulted in the two-subunit structure, TRAP 5b[172]. Later it was investigated with cysteine proteinases and MMPs, which are more likely the physiological enzymes found in an OCs microenvironment. It was shown that the proteolytic processing of the flexible loop with cysteine proteases like CtsK, L and B resulted in TRAP enzyme activation, similar (CtsK/L) or slightly lower (CstB) to the endogenous TRAP derived from OCs. CtsK is the most abundant cysteine protease in OCs and is currently the most efficient protease known to activate TRAP by proteolytical processing[171]. However, there are other as well, i.e. CstL, with an approximately the same efficient as CtsK but is present in non-OCs in bone[171] and therefore not likely to cleave TRAP 5a in OCs.

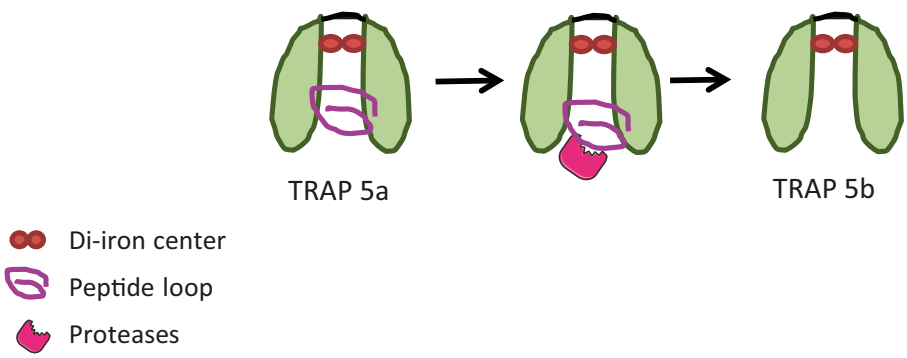

Figure 9. Proteolytic cleavage of TRAP 5a generates TRAP 5b. Proteases and/or MMPs, particularly CtsK in OCs, cleave the flexible peptide loop of TRAP 5a and it generates the TRAP 5b dimer-form stabilized by a disulphide bond. The cleavage leads to changes in the ferric active center increasing the phosphatase activity of TRAP 5b.

MMPs have also been reported to cleave TRAP to some extent, however it results in an inefficient enzyme activation[171]. Ctsk$^{-/-}$ mice revealed that in absence of CtsK another protease was compensating for its function and TRAP 5a was still cleaved to the same extend to TRAP 5b, but there was an accumulation of uncleaved TRAP 5a when was compared to the wild type[170], indicating that there are different pathways or subpopulations of TRAP, where some TRAP is cleaved intracellularly and some depends on CtsK activity and accumulates. Altogether, proteolytic cleavage by cysteine proteases, specially CtsK, was proposed as a mechanism for the enzyme activation of TRAP[173].

Further studies revealed that TRAP 5a has a pH optimum of 5.0-5.2, whereas TRAP 5b has a pH optimum of 5.8-6.9. Moreover, TRAP 5b exhibit ~10X higher activity than 5a[166,174,175] and a higher affinity for OPN and NPP substrates[171,172].

TRAP 5a and 5b can be separated and purified using heparin ion-exchange FPLC [164,170,171,176,177]. It was shown by Ljusberg et al., that bone TRAP derived from mouse and rat exhibited an elution profile with three separable peaks where the first eluted peak 1 represent the monomeric TRAP 5a form and major peaks 2 and 3 represent proteolytically cleaved TRAP 5b isoform[170,171].

Using a monoclonal antibody specific for TRAP 5a, Lång et al, found that in general, TRAP 5a has a broader expression pattern than TRAP 5b[171,178]. In particular, cells outside the hematopoietic lineage seem to express predominantly TRAP 5a while in cells derived from the hematopoietic lineage (e.g. OCs, macrophages) both isoforms were found to be express at different ratios.

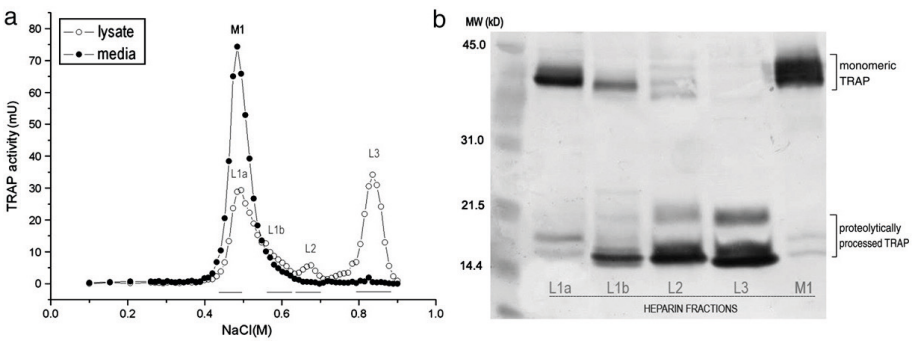

Figure 10. Cleavage profile analysis of intra and extracellular TRAP by Heparin FPLC and western blot. (a) Elution profile of TRAP activity of lysate and media by MDA-C11 cells (b) western blot analysis of Heparin FPLC elution peaks . Reprinted with permission of Zenger, S., Ek-Rylander, B. *Biogenesis of tartrate-resistant acid phosphatase isoforms 5a and 5b in stably transfected MDA-MB-231 breast cancer epithelial cells*[15]. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2010;10:598-607. Copyright © Elsevier.

Redox activation

The redox-state in also an important regulator of TRAP enzyme activity and the reduction of iron ions increases also the phosphatase activity[179]. The TRAP active site contains two metal-bindings domains, coordinated by a cage of critical residues coordinating the catalytic center which is composed of a fixed Fe^{3+}(ferric) and a redox active ion of Fe^{2+} (ferrous) in humans[180,181]. When reduced (Fe^{3+}/Fe^{2+}), TRAP is enzymatically active and when oxidized (Fe^{3+}/Fe^{3+}) e.g. by treatment with hydrogen peroxide, TRAP is enzymatically inactive[182].

It was proved by Lång et al, that the enzyme activation is a two-step process, where the proteolytic cleavage of the peptide loop is necessary for a secondary reduction of the active site, and if the cleavage doesn't occur the reduction of the enzyme is less efficient[183].

3.2 TRAP in bone

Studies on $TRAP^{-/-}$ mice showed a mild osteopetrotic phenotype with an extended area of metaphyseal mineralized cartilage with equal amounts of OCs that form smaller pits on dentine but produce equal amount of CTX fragments [169,184]. Further, the $TRAP^{-/-}$ mouse have disturbed vesicular transport in OCs[81]. On the other hand, mice over-expressing TRAP were mildly osteoporotic with a conflicting results between groups. While Angel et al., in 2000 showed a decreased trabecular density in long bones[185], Gradin et al., in 2012 showed no difference in the trabecular bone volume but instead increased BMC and BMD[186]. Gradin et al., also showed that TRAP expression in OB and OY was increased in the TRAP overexpressing mouse and that this was coupled to increased expression of OB markers suggesting an anabolic effect of TRAP on bone[186].

3.2.1 TRAP knockout mice

The role of TRAP in bone remodeling has been elucidated by the TRAP knockout mice[187], which nevertheless lacking TRAP activity, are not apparently sick, and viable under laboratory conditions. However, these mice suffer from deformity of the long bones and axial skeleton due to OCs defects. Both tooth eruption and development of skull and flat bones is normal, indicating a role of TRAP in endochondral ossification [169]. Adult TRAP KO mice have higher bone mineralization density and an increased cortical content, reflecting a mild osteopetrosis as early as 4 weeks of age.

The formation of OCs is normal[169], however, bone resorption is defective[184]. Examination with TECM revealed that these OCs exhibit an extended area of RB and tend to accumulate cytoplasmic vesicles[169] containing filamentous material[188]. Because the location of CtsK is normal, the accumulation of intracellular vesicles is probably not due to an impaired secretion. Different explanations are available, but none is completely proved. 1) Because the ROS function in TRAP KO is defective, they internalized bone degradation products and vacuoles accumulating filamentous

material into the cytoplasm instead of being released by transcytosis and secreted to blood circulation through the FSD[189]. 2) TRAP reduced dephosphorylating function modulating intracellular vesicular transport is probably impaired[169].

3.2.2 TRAP localization and function in OCs

Localization and secretion

In OCs, TRAP 5a is found in intracellular vesicles. These vesicles are then fused with the RB[159] and TRAP 5a released into the resorption lacuna where it is cleaved extracellularly by CtsK to produce TRAP 5b, and consequently, is then localized in the RB and in the resorption lacuna[171,190].

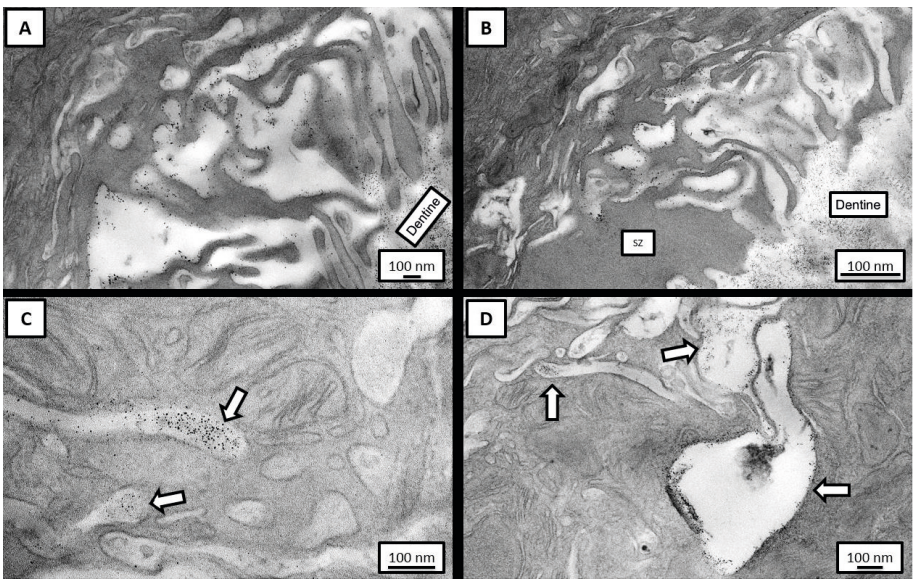

Figure 11. Morphological characterisation of TRAP-positive vesicles in resorbing osteoclasts using pre-embedding immunoEM (nanogold + silver enhancement). Mouse osteoclasts cultured on dentine were processed for TEM. Positive labelling was identified as an organelle with at least 4 gold particles. (A&B) TRAP localized extracellularly to the surface of the resorption pit and along the membrane of the ruffled border. (C&D) TRAP-positive vesicles (arrows) were found intracellularly, contained electron dense material often surrounded by a haloed region and single membrane. The vesicles measured about 300 nm in diameter and had a rounded or elongated morphology. This picture belongs to Emma McDermott thesis titled '***Characterisation of the osteoclast ruffled border using advanced imaging thecniques***' from the University of Abeerden (2017) and hasn't been published yet. Protocols for staining and sample preparation can be found there. *By kind permission of E. McDermott and M.H. Helfrich, University of Aberdeen.*

Although ctsk and TRAP are confined in the same cell[191], their secretion during resorption seems to be different[170]. TRAP 5a is secreted after CtsK, suggesting a role for TRAP 5b in the later migration process after collagen degradation[190]. During resorption, TRAP 5b from the resorption lacuna is endocytosis and transported to the FSD domain through the transcytotic pathway, together with collagen degradation products.

Moreover, TRAP 5b can also be cleaved intracellularly in endosomal vesicles originating from the Golgi network[170] where an intermediate form of TRAP – cleaved by an unknown protease- can also fuse with the transcytotic vesicles.

TRAP as a regulator of adhesion and migration

OPN is a glycoprotein capable of mediating OC attachment to bone matrix through $a_v\beta_3$-integrin integrin[192]. It is the only identified in vivo substrate of TRAP, where TRAP 5b modulates OPN by dephosphorylation[173], with functional effects on i.e. OCs migration[41]. It has been reported that OC derived from wild type, when in contact with TRAP dephosphorylated OPN does not induce OC attachment or migration[192]. Interestingly, OCs derived from TRAP overexpressing mice seeded on phosphorylated OPN showed higher migration rates than wild type OCs[41].

It has been reported that during bone remodeling the resorption area is cemented by a line of OPN[48]. TRAP 5b is coincidentally secreted in the leading edge of a resorptive OCs[193] and can dephosphorylate OPN. In general, phosphatases have a repellent effect, as they are full of negatives charges, they can change the conformation of the protein and affect the attachment through integrins. Some integrins like $a_v\beta_3$-integrin care about the phosphate status, but others like $a_v\beta_5$-integrin or CD44 are not affected by the phosphate status[194]. This implies that OCs may control the homing of cells to the resorption lacuna and that changes in the secretion of TRAP 5b can lead to different pathologies e.g. inflammation and tumor progression.

Lastly, a genetic disease, causing loss of function in the ACP5 gene increases the amount of phosphorylated OPN, resulting in the autoimmune phenotype spondyloenchondrodysplasia (SPENCD)[195], characterized by skeleton abnormalities and autoimmune manifestations (Details in section 3.3).

TRAP and trafficking of lysosomal enzymes

TRAP 5b has also been suggested to be responsible for dephosphorylation of mannose-6-P (Man-6-P) on lysosomal proteins[196], a recognition marker labeled by the Golgi network, that marks proteins that are going to be transported to the lysosomes or endosomal vesicles, thus controlling vesicle trafficking.

Vesicular trafficking in OCs

Studies on the TRAP knockout mice[169] showed a cytoplasm accumulation of vesicles due to a disturbance in the RB and a defective intracellular transport of vesicles, suggesting a novel role of TRAP in the modulation of intracellular vesicular transport in OCs.

TRAP in collagen degradation (ROS)

In addition to its phosphatase activity, TRAP 5b has also been suggested to be involved in bone matrix degradation by producing reactive oxygen species (ROS)[189,197,198] where TRAP 5b containing vesicles fuse with vesicles containing remnants from resorption and further degraded them thorough the formation of ROS generated by TRAP during transcytosis.

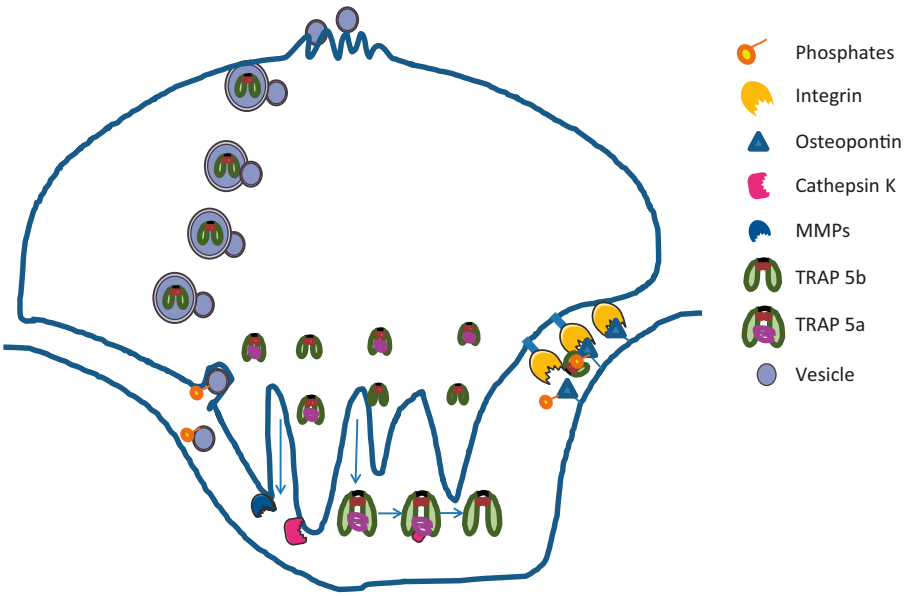

Figure 12. Major function of TRAP as a phosphatase. TRAP may come into direct contact with CtsK which could proteolytically activate TRAP at its site of action. 1) OC attachment to the bone matrix is mediated through the interaction of the integrins ( ) and phosphorylated OPN ( ). TRAP 5b is able to dephosphorylate the OPN and allows the OC to move. **2)** CtsK and MMPs are released to the resorption lacuna where TRAP 5a is processed to TRAP 5b by CtsK. **3)** Bone degradation products (organic and inorganic) are endocytosed from the RB and transported in vesicles through the cell to the FSD, where they are released into the bloodstream. TRAP 5b can also be found intracellularly and is involved in vesicle degradation (ROS) and vesicle trafficking (via transcytosis pathway). Vesicles containing TRAP 5b fuse with endocytic vesicles containing bone degradation products and are degraded through the phosphatase reaction. TRAP 5b also dephosphorylates mannose-6-P, which is a signal for transporting proteins to the endosomal vesicles, recycling it.

3.2.3 TRAP as a growth and differentiation factor

Most studies analyzing the role of TRAP as a growth and differentiating factor have been carried out in OB, showing that TRAP is involved in OB. It was previously shown that OC-derived TRAP localized to the resorption lacuna, which may interact with pre-osteoblastic cells and could serve as a coupling regulator of bone formation, mediating the recruitment and activation of OBs[186,200,202].

It has been shown in adipocytes, which are derived from the same stem cell lineage as OBs, that TRAP 5a, but not 5b, which is secreted by adipose tissue macrophages can induce hyperplastic obesity in mice[203]. TRAP 5a also increases differentiation of human adipocytes (3T3-L1) *in vitro*, suggesting a regulating adipogenic role of TRAP 5a[204]. It has also been shown that TRAP 5a interacts with components of the ECM and exhibits growth factor-like activities for pre-adipocytes and mesenchymal stem cells affecting its proliferation, and differentiation[203,205].

3.2.4 TRAP in OBs and OYs as a coupling factor

Solberg et al., (2014) reported that TRAP containing vesicles co-localized with RANKL and OPG in OB vesicles[206], indicating that either vesicular TRAP may have a role intracellularly in the vesicles or a role extracellularly where RANKL/OPG and TRAP could be released together. This implies that TRAP has a direct role coupling bone resorption and bone formation during the bone remodeling process.

TRAP was shown to interact with TGFβR-interacting protein-1 (TRIP-1), an intracellular protein expressed in OB with high affinity for TRAP 5b, what has been proposed as a bridge between TGFβ and TRAP[207].

When TRIP-1 was released via exosomes to the ECM it was shown to induce mineralization upon binding with collagen type 1[208]. TRAP was shown to interact intracellularly with TRIP-1, activating the TβRII and inducing OB differentiation after resorption[209]. Furthermore, TRIP-1 knockdown abolished OB differentiation and proliferation[209].

3.2.5 TRAP as a mineralization promoter

Bone alkaline phosphatase (BALP) is a glycoprotein located to the outer surface of cells and matrix vesicles. It is an important component in hard tissue formation and it appears to control mineralization through regulation of the phosphorylated stated of OPN. It functions as a mineralization promoter by increasing the concentration of inorganic phosphate through dephosphorylation of OPN[210]. BALP can also inhibit the formation of mineral by decreasing the extracellular concentration of pyrophosphate. Both BALP and TRAP can alleviate the inhibitory effect of OPN on mineralization, suggesting that TRAP could substitute for

alkaline phosphatase as a mineralization promoter. When the phosphatase activity between BALP and TRAP was compared in vitro via dephosphorylation of bovine milk OPN, TRAP showed the highest activity, as it was able to remove all the 23 phosphates of OPN[211].

3.3 Proposed functions of TRAP outside bone

TRAP expression can be detected in other cell types such as neurons[212], hepatocytes and keratinocytes[15,213–215], macrophages and dendritic cells[216].

TRAP in autoimmunity and inflammation

The rare recessive human genetic disease spondyloenchondrodysplasia (SPENCD) which is associated with short stature, skeletal sclerosis, brain calcifications and various forms of autoimmunity, was shown to be caused by inactivating mutations in the human TRAP gene causing an increase of the ratios on phosphorylated OPN[217,218]. TRAP inactivation in SPENCD is associated with OPN hyper phosphorylation. Phosphorylated OPN is known to increase macrophage and OC activation through increasing the basal expression of TH1 cytokines e.g. TNF-γ and IL-1 and thus modulating an inflammatory response.

During inflammation, phosphorylated OPN triggers the immune system by inducing macrophages, dendritic cells, monocytes and T cells to accumulate at the site of injury and also function as a Th1 type cytokines e.g. via stimulation of the CD44 co-receptor, mediating in chronic inflammation and autoimmune diseases like SPENCD.

TRAP expression in phagocytic cells is associated with an enhanced bacteria clearance capacity, whereas mice overexpressing TRAP had elevated levels of intracellular ROS[219] and macrophages lacking TRAP failed to remove phagocytosed bacteria[187]. $Acp5^{-/-}$ mice have also an increased basal expression of inflammatory cytokines such as IL-1, IL-12 and TNFα while dendritic cells lacking TRAP have an increased expression of IL-10 but reduced response to T-dependent antigens[187,220].

TRAP in cancer

Elevated TRAP 5b serum levels have been reported in malignant diseases involving bone e.g. multiple myeloma, bone metastasis of breast and prostate cancer[163]. TRAP has been proposed as a driver of cancer metastasis in breast cancer cells through regulation of CD44, an important protein implicated in the regulation of mesenchymal epithelial transition[221]. Forkhead box M1 (foxM1) is a transcriptional regulator of TRAP that was found to promote tumor metastasis and its presence indicates a poor prognosis in hepatocellular carcinoma.

3.4 TRAP as a biomarker

TRAP 5b correlates to OC number

TRAP 5b is mainly secreted by polarized OCs and correlates to OC number/bone resorption, thus it correlates also to metabolic bone diseases [179,222,223].

Halleen et al., purified TRAP from human osteoclasts and they used it to produce a monoclonal antibody O1A against osteoclastic TRAP[223]. They realized that the antigenic properties and pH optimal for TRAP derived from OCs was identical to the previously isolated TRAP 5b detected in serum and it was then hypothesized that TRAP 5b was secreted by OCs. A strong correlation of secreted 5b with OC number was found in mouse[224]and human[193] OCs cultured on plastic (i.e. non-resorbing OCs).

It was previously described by Stepan et al. (1983), that plasma TRAP activity was continuously reduced after removal of parathyroid adenomas from patients with primary hyperparathyroidism[225]. Several studies have shown that changes in bone resorption are often associated with changes that modulate OCs numbers e.g. hormonal changes, treatments, and higher levels of TRAP 5b have been measured in postmenopausal osteoporosis[163], breast and prostate cancer[226] renal bone diseases, multiple myeloma and Paget's disease. Moreover, TRAP 5b values can predict the risk of future fractures[227,228]. TRAP 5b loses its iron content and it is cleared through the liver. Therefore secreted TRAP 5b describes the amount of intact 5b that has been secreted by OCs at a certain time reflecting the number of OC. TRAP 5b also correlates with OCs number in patients with autosomal dominant osteopetrosis type 2 (ADO2)[229], various osteopetrotic rat strains[229] and human OC cultures[224]. These results seem to point out that TRAP 5b is being secreted from OCs at a constant rate despite the resorbing activity of the cells, correlating to the amount of OCs but not to resorption.

TRAP 5b serum levels were found to be significantly increased in patients with aseptic loosening, however results didn't correlate with CTX-I measurements, indicating that TRAP 5b could help to monitor cases of hip replacement for aseptic loosening[230].

TRAP 5a as a marker of chronic inflammation

Serum TRAP 5a is highly secreted by macrophages and dendritic cells, has non-phosphatase activity, and was considered an inactive isoform for many years. Nowadays its role as an inflammatory biomarker has been described in different pathologies. It increases its concentration in patients with chronic inflammation diseases such as systemic lupus erythematosus (SLE)[231], rheumatoid arthritis[175], atherosclerosis[232], sarcoidosis[233] and is a potential marker for disease severity in

cardiovascular and kidney disease cancer progression and treatment resistance[234]. Moreover, TRAP blood or serum levels are often used as a reference index for obesity and bone mass density (BMD)[203,235] and applied to monitor breast cancer metastasis[226] but the exactly mechanism is still not known. In metabolic bone diseases 5a correlates with inflammatory markers[236]

3.5 Measurement of TRAP isoforms

Due to disease-specific isoform association and the different biological roles of TRAP 5a and 5b[213,237] there is a need to measure TRAP 5a and 5b serum protein levels in the same sample in order to assess (1) changes in ratios of the isoforms and (2) correlation between different conditions and both isoforms. In a clinical setting, this facilitates detailed diagnosis, monitoring the severity or the development and provides a prognosis for different conditions. Today, there are different TRAP assays commercially available measuring total TRAP or TRAP isoforms separately. Their chemistry is based either on an immuno-immobilization activity assays, (IIAA) capturing total TRAP or specifically TRAP 5a and then measuring enzyme activity at respective pH optima for the two isoforms[224,238,239], or sandwich ELISA measuring TRAP 5a or total TRAP concentration[240–242].

For TRAP 5a, there are very specific sandwich ELISAs that are sold, but there are no reports of antibodies specific to TRAP 5b. TRAP 5b has been evaluated by measuring total TRAP and then the enzyme activity, which is proven to measure OCs number[243]. One ELISA is the bone TRAP assay based on a pH selection (SBA-Sciences, Oulu, Finland)[242] that uses an anti-TRAP antibody, O1A, that binds the enzyme and the pH is then swiftly changed to pH 5.0-5.2, where the TRAP 5b is found to be more active. The other assay that measures TRAP activity is one using the selective and preferred substrate for TRAP, naphthyl ASKI phosphate[244]

However, the typically more abundant TRAP 5a is able to interfere in these assay types and this requires calculation steps to estimate the actual TRAP 5b concentration[245]. Moreover, the redox state of the TRAP active site influences enzyme activity and therefore TRAP enzyme activity may not reflect the amount of TRAP protein in the sample.

3.6 TRAP inhibitors

A number of TRAP inhibitors have been identified by inhibiting TRAP activity[246]. However, these kind of selective inhibitors are usually weak, toxic and lack drug-like characteristics. Oxidizing agents such as hydrogen peroxide[212] inactivate the enzyme via conversion of the $Fe2^{+}$ ion to a $Fe3^{+}$.

Au[bipyOMe]

Gold compounds have been used in clinics since the 70s to treat inflammatory conditions such as rheumatoid arthritis. However, gold is itself redox active and thus the gold compounds are highly unspecific inhibitors due to their oxidative activity. In this respect, there is a high change AubipyOMe reducing the active site of TRAP or acting on other proteins in the cell as well. Given that the mechanism of inhibition was never fully understood and several side effects were observed, its application was discontinued[247,248]. Nevertheless, its application in research as TRAP inhibitors is valuable. The applicability of the gold coordination compound AubipyOme to inhibit TRAP activity was recently shown in alveolar macrophages[249], where it was conceptualized as an aerosol formulation to minimize the systemic effects of inflammation in lungs.

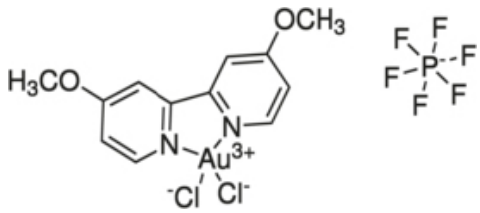

Figure 13. Chemical structure of the Au(III) compound [Au(4,4′-dimethoxy-2,2′-bipyridine) Cl2][PF6] (AubipyOMe).

5-phenylnicotinic acid

Scwender et al. screened a series of phosphate compounds against TRAP and found that small compounds can inhibit TRAP by mimicking substrates[250], which is the type of inhibitor that was used in paper IV. 5-phenylnicotinic acid (5PNA) is a small chemical compound of 200 Da that was found to block TRAP 5b activity[251]. It is a competitive inhibitor that takes the place of the substrate. However if the activity is mediated by receptor binding it may not inhibit the activity[251], although the binding was suggested to be at the active site through docking studies was never empirically tested[252].

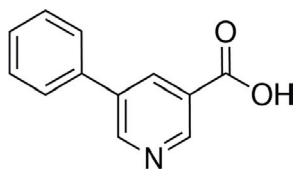

Figure 14. Chemical structure of the 5-phenynicotinic acid ($C_{12}H_9NO_2$)

4 AIM OF THE THESIS

The overall aim of this dissertation was to investigate the role of TRAP isoforms in bone remodeling focusing on TRAP in OCs. The aim can be further sub-divided into the following objectives:

- In order to measure the protein levels of TRAP 5a and 5b isoforms and evaluate the role of TRAP 5a in differentiation OCs, we developed an applicable sandwich ELISA that can detect both isoforms separately from the same sample. This assay allowed us to elucidate more about the role of 5a besides of being a precursor for TRAP 5b as well as protein levels of TRAP 5b secreted from in osteoclasts (**Paper I**).
- Investigate the relationship of TRAP isoforms and the known substrate for 5b, OPN, in inflammatory bone diseases and the potential application of TRAP 5a, 5b and 5b/5a as a disease progression marker (**Paper II and I**).
- Study of the heterogenetic RAW264.7 macrophage cell line and characterize a more stable subclone with higher capability to form TRAP positive OC-like cells (**Paper III**).
- Study the biological role of TRAP isoforms during osteoclast differentiation under physiological and pathological conditions by applying the small molecule inhibitor, 5PNA in a RANKL and IL4/IL13 –induced systems (**Paper IV and I**).

5 REMARKS ON METHODOLOGY

5.1 Ethical considerations

Human peripheral blood monocytes (hPBMCs) were isolated from buffy coats of donated blood of healthy men at Karolinska University Hospital Laboratory, Huddinge, Sweden. The surplus blood products were coded for research use. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Karolisnka Institutet and the Swedish Research Council. Written informed consent was obtained from all individual participants included in the study in accordance with the Helsinki Declaration.

5.2 Antibodies recognizing total TRAP and TRAP isoforms

The structural difference between TRAP 5a and TRAP 5b is the loop region present in 5a but absent in 5b[253]. Thus, it is possible to raise antibodies against TRAP 5a using either the loop region as immunogen or screening for TRAP 5a specific Abs raised against the whole TRAP 5a protein. However, it is not possible to raise TRAP 5b specific Abs. Therefore, in this thesis we use a combination of anti-TRAP 5a Abs and anti- total TRAP Abs to determine the protein concentration of the two isoforms.

Monoclonal mouse antibodies against TRAP 5a and total TRAP was made by Mabtech AB, Nacka, Sweden[205]. Briefly, mAb against TRAP 5a was raised using the human loop peptide region 165 to 177 aa from the 325 aa TRAP sequence (UniProt P13686) as immunogen. mAb against total TRAP was raised against recombinant human TRAP 5a. Antibodies used for ICC, rabbit anti rat total TRAP and rabbit anti mouse TRAP 5a, were raised in rabbits against recombinant rat TRAP 5a and a peptide representing the mouse loop domain aa 167-183 in the mouse TRAP sequence (NP 031414), respectively, by Innovagen AB, Lund, Sweden[183]. Specificity of TRAP 5a antibodies was verified using Western Blot and ELISA against TRAP 5a and 5b.

5.3 The double TRAP 5a/5b sandwich ELISA

A previous published TRAP 5a ELISA was used as base to develop the double TRAP 5a/5b sandwich ELISA (Figure 15)[235]. The new double ELISA uses three antibodies: mAb 46, specific for TRAP 5a[235] and mAb 25.44 recognizing both TRAP 5a and TRAP 5b. mAb 12.56 also recognizes both isoforms, is used as a detection antibody and is biotinylated. The monoclonal antibody specific for TRAP 5a was developed using the loop present in TRAP 5a but absent in TRAP 5b due to proteolytic cleavege[165]. In order to separate TRAP 5a from TRAP 5b, the method was designed as follows: In the first step, sample was added to TRAP 5a specific antibody, i.e. mAb 46 coated wells and TRAP 5a was captured during overnight incubation at +4°C and subsequently measured using biotinylated mAb 12.56 and streptavidin

conjugated HRP. After overnight incubation the sample, now depleted of TRAP 5a but having the original amount of TRAP 5b, was transferred to wells coated with mAb25.44, detecting both isoforms, and the remaining TRAP 5b concentration was measured using biotinylated mAb 12.56- and biotin/streptavidin conjugated HRP.

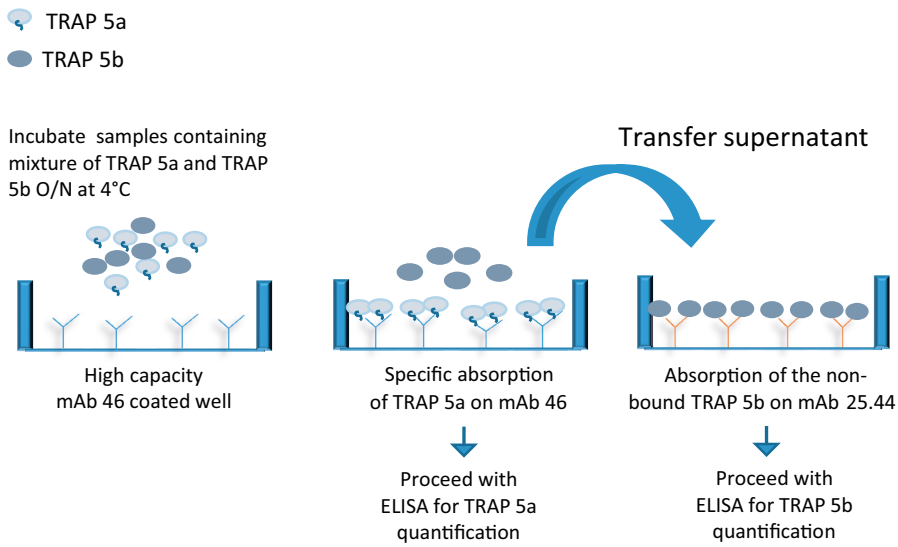

Figure 15. TRAP 5a and TRAP 5b separation principle in the double TRAP 5a/5b sandwich ELISA.

5.4 RAW 264.7 cell line

The murine monocyte/macrophage cell line RAW264.7 has been extensively used to study bone biology as it is able to respond to RANKL and form TRAP multinucleated cells, resembling osteoclast (OCs)[254]. RAW 264.7 was established from a tumor induced through injection of Abelson murine leukemia virus into a BALB/c mice[255]. RAW 264.7 cells endogenously synthesize M-CSF, hence RANKL alone is sufficient to drive their differentiation into OC-like cells[256]. RAW 264.7 has several advantages[254], however, one disadvantage is that it is a very heterogeneous cell line[257] than can make experiments difficult to replicate.

5.5 CD14+ monocytes isolation

We positively separated CD14+ cells based on the expression of the CD14 antigen. Magnetic cell sorting of human leukocytes was used to isolate the CD14+ monocytes from buffy coats (see section 5.1 Ethical permits). Briefly, cells were

magnetically labeled with Microbeads, conjugated with a monoclonal anti-human CD14 antibody. CD14+ are retained in a magnetic column while the rest are flushed away. Later the CD14+ cells are eluded after removal of the column from the magnetic field.

5.6 Animal and cell models used in these projects

Mice are increasingly used in investigation of skeletal growth and regulation because they are biologically similar to humans. Mice can be genetically manipulated, have an accelerated life span, are cost-effective and easy to breed. Similar to humans, murine bone physiology consists also on constant bone modelling and remodeling. Unlike humans, bone acquisition and bone growth continues after sexual maturity, which is around 6-8 weeks of age[258]. In mice the peak bone mass is achieved between 4-6 months of age[258,259].

5.6.1 TRAP knock-out mice

TRAP-deficient mice were generated by Allison Hayman and Timothy Cox[184] in collaboration with Martin Evans at Cambridge University. Mice were provided by Paul Saftig's lab from Kiel Universität to Arne Egesten from the University of Lund. ACP $5^{-/-}$ are not apparently sick, they present short limbs, and white teeth, probably due to over mineralization, are mildly osteopetrotic and have lost hearing[169,260].

We used female and male mice that were 7-11 weeks of age at the start of the experiment.

5.6.2 C57BL/6N mice

Inbreed strains of mice, such as C57BL/6N mice, originally from Charles River Laboratories, Germany, allow for genetic uniformity of each animal, allowing reproducible results with smaller samples sizes. Breeding was done in the Facilities of Sahlgrenska Academy Institute of Medicine, University of Gothenburg by Professor Ulf Lerner and Petra Henning. We used male mice that were 8-12 weeks of age for isolation of bone marrow macrophages from femur and tibia

We used CD45.1+ on C57BL/6 genetic background, donated from Hong Qian, Karolinska University as a control for the TRAP knock out mice. Mice used were between 8-11 weeks.

6 RESULTS AND DISCUSSION

6.1 Paper I

Aim

In this paper we aimed to develop a human TRAP 5a and 5b isoform specific ELISA that would give the possibility of quantifying separately TRAP5a and TRAP 5b protein levels in different types of samples i.e. serum, cell culture media and cell lysate. This human TRAP 5a and 5b isoform specific ELISA was then used to investigate TRAP 5a/5b levels and correlations in healthy men (paper I) as well as production and secretion of TRAP 5a/5b in in vitro cultured OCs (paper IV).

Double TRAP 5a/5b sandwich ELISA development

We were able to develop a double TRAP5a/5b sandwich ELISA that can be used to measure human TRAP 5a and 5b protein levels in serum, cell culture media and cell lysate. However, in serum, TRAP is found in complex with alpha 2 macroglobulin (a2M), which affects the composition of the active site[261] and loop region i.e. the antigen binding part of TRAP 5a. We therefore hypothesized that serum TRAP 5a would not bind efficiently to mAb46, as has been shown for the TRAP 5a ELISA[235] and that this would result in low TRAP 5a detection on mAb46 and a consequent higher TRAP 5b measurement on the mAb25.44 due to high TRAP 5a carry-over. When serum samples were pre-treated in order to change the pH and allow any complex involving TRAP to disassociate, TRAP 5a detection increased and TRAP 5b detection was decreased, being more similar to previously reported TRAP isoforms measurements[262].

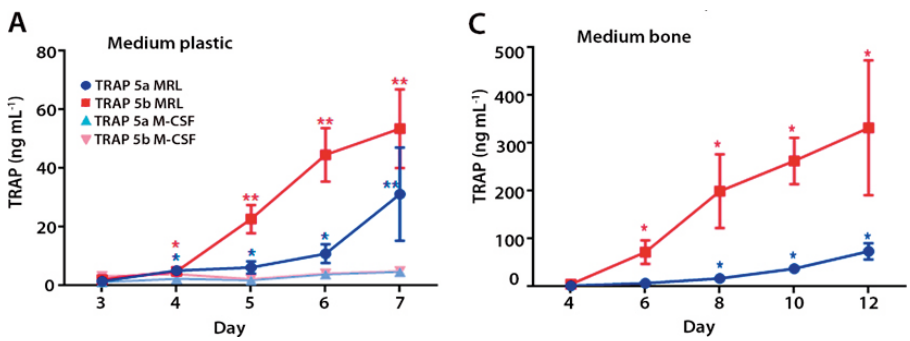

Figure 16. Measurement of TRAP 5a secretion from CD14+ cells induced with RANKL and M-CSF. A) cells cultured on plastic secrete both isoforms from day 4 C) TRAP isoforms secreted when cells are cultured on bone slices. There is a delay in TRAP secretion being significant vs when cultured on plastic.

Validation of the TRAP 5a/5b double ELISA showed that parameters e.g. inter- and intra-assay variations were within acceptable ranges, however, when/if the ELISA is subject for commercialization/diagnostic use in laboratories, validation in accordance with the FDA guidelines for method validation, "Bioanalytical Method Validation Guidance for Industry" will be applied.

TRAP 5a and 5b protein in serum from healthy men and correlation to CTX-I

In previous studies, human serum, TRAP 5a and 5b protein have been separated by chromatography, identified by electrophoresis and quantified by a specific ELISA[223] . These studies showed that TRAP 5a accounted for 87% of the enzyme protein but 55% of total the activity[262]. Separation with chromatography showed that serum TRAP consisted of ~87% and TRAP 5b ~13%[262]. This is comparable to 75% vs 25% for TRAP 5a and 5b protein in human serum measured with the double-TRAP 5a/5b sandwich ELISA.

In serum from heathy men, there was a positive correlation between TRAP isoforms 5a and 5b. Surprisingly, not only 5b, but also 5a correlated positively to resorption marker CTX-I. This suggests that both isoforms are secreted from differentiating OCs during normal bone turnover. However, 5a is also derived from other sources e.g. macrophages and the positive correlation with CTX-I seen here in serum from healthy individuals likely indicates that the part of 5a derived from non-OC sources is constant. In an individual in a pathological state, e.g. inflammation, the pool of 5a originating from other sources is likely to be affected and therefore the correlation with CTX-I might disappear.

It was observed that the TRAP isoforms in serum correlate with the resorption marker- Collagen type-I C-terminal fragment (CTX) in healthy individuals (paper I), while in case of inflammatory conditions TRAP5a has been shown to correlate with certain inflammatory markers e.g. YKL-40 and IgM RF but not with the resorption markers in TRAP 5a have not been studied before[263]. In pathological situation i.e. anorexia, the correlation between TRAP 5a and TRAP 5b is lost, and could indicate a sign of a pathological bone disorder[264]. Both tartrate-resistant acid phosphatase isoforms 5a and 5b are secreted by differentiating osteoclasts and correlate with serum levels of CTX-I in men.

Distribution of TRAP 5a and 5b in in vitro cultured OCs

TRAP activity staining confirmed the appearance of multinucleated TRAP positive cells with varying activity levels. By ICC, TRAP 5a specific staining was apparent in many of the multinucleated and mononuclear cells, while total TRAP signal was lower in larger multinucleated cells, indicating effective secretion of the TRAP 5b isoform. A few mononuclear cells stained double positive for both isoforms,

indicating that TRAP processing starts already at a late mononuclear stage and could be a marker for mononuclear pre-OCs and since it is, produce it could have a role in the mononuclear pre-osteoclasts.

Production and secretion of TRAP 5a and 5b from in vitro cultured OCs

There is a common perception that TRAP is secreted by OCs as TRAP 5a, later is proteolytically processed extracellularly by e.g. CtsK to produce TRAP 5b, before being endocytosed, and transported through the OC in transcytotic vesicles where is finally secreted through the FSD domain[15]. However, data obtained here showed that secretion of both TRAP 5b as well as TRAP 5a isoform was progressively increasing, although 5b dominated. Overall it indicates that OCs contribute to TRAP 5a levels in serum, where it was previously indicated that only macrophages and dendritic cells contributed.

Further, both isoforms were also present in cell lysate before either of the isoforms was observed in medium, further establishing that 5b can be formed intracellular in OCs where TRAP 5b may have a role in mononuclear pre-OCs, although it is not clear if this pool of 5b can also be secreted from OCs[15].

Conclusion

- A new double TRAP 5a/5b sandwich ELISA measuring TRAP 5a and 5b protein in the same sample was developed and verified for use with serum, cell culture media and cell lysate. The difference observed with this new TRAP 5a/5b sandwich previously published IIAAs shows the importance of measuring TRAP 5a and 5b protein.
- In vitro cultured OCs produce and secrete both TRAP 5a and 5b. TRAP 5a and 5b correlate to each other in both serum from healthy males and in vitro cultured OCs. TRAP 5a correlates to CTX-I in both healthy males and in vitro cultured OCs on cultured on bone. This suggests that a considerable proportion of serum TRAP 5a originates from OCs and that it might show an undiscovered regulatory mechanism between bone resorption and bone remodeling.

6.2 Paper II

Aim

The aim was to assess the relation of OPN phosphorylation with TRAP 5a and 5b protein in OA and RA joint tissues, in order to possible distinguish differences between these different diseases characterized by cartilage and bone destruction

Phospho-OPN and TRAP in synovial fluid

Full-length OPN was higher in RA+/- patients compared to OA patients. This was also true for phosphorylated C-terminal end of OPN fragment.

It was possible to detect TRAP 5a and TRAP 5b protein in synovial fluid although two patients had undetectable TRAP 5b levels. TRAP 5a was higher in RA+ compared to RA- and OA, while TRAP 5b was higher in OA compared to RA+/- groups. However, total TRAP was the same in all three groups.

Interestingly, the TRAP 5b/5a ratio was higher in OA compared to RA and it correlated negatively to the amount of phosphorylated OPN i.e. the less TRAP 5b the more phosphorylated OPN. This is probably due to the high individual variation.

Conclusions

- It is possible to use the double TRAP 5a/5b sandwich ELISA to measure TRAP 5a and 5b protein in synovial fluid.
- Analysis of the TRAP 5b/5a ratio was more sensitive than measuring TRAP 5a and 5b alone in establishing differences between the different groups.
- Decreased ratio of TRAP 5b/5a in RA patients was associated with an increased amount of phospho-OPN strengthening previous data showing that OPN is an vivo substrate for TRAP 5b.

6.3 Paper III

Aim

The aim of this study was to isolate RAW 264.7 clones more specifically destined to form OC-like cells and clones with a low ability to form OC-like cells. In addition, the study aimed to investigate why RAW264.7 have different capacity to form OC-like cells and understand the hallmarks of decision points in differentiation of these cells to OC or macrophage direction.

Selection of subclones

First, subclones were isolated from RAW 264.7 parental cells by single cell cloning, two clones, H9.2d5 (alias H9) and J8.2g6 (alias J8), were selected for further analysis based on the expression levels of two OC markers, TRAP and CtsK. H9 exhibit high TRAP and CtsK mRNA expression while J8 had very low mRNA expression of both these markers.

H9, but not J8, can form OCs in vitro on plastic and bone

RANKL treated subclone H9 had a variety of OC related features i.e. increased acidification, pit formation and CTX-I formation. On the other hand, phagocytosis, a macrophage characteristic, were decreased. RANKL treated H9 also exhibited increased gene expression of OC target genes i.e. TRAP, ctsk, OC-STAMP, ATP06vd2 and ATP6i. On the other hand, subclone J8, did not seem to be able to exhibit any typical OC characteristic.

H9 forms OCs faster than RAW 264.7 due to higher nuclear translocation of Nfatc1

Interestingly, H9 formed OC-like cells faster than parental RAW 264.7 cells although their capacity to function as OC-like cells did not differ after 4 days of RANKL-treatment. Gene expression analysis revealed that unstimulated H9 express higher levels of OC master transcription factor NFATc1 compared to RAW 264.7. NFATc1 ICC further revealed that nuclear translocation in H9 is higher suggesting that H9 therefore more efficient in rapid expression of OCs related genes.

Conclusion

- Sub-clone H9 is more homogenous and committed OC precursor than the general RAW 264.7 cells.
- RANKL differentiation of H9 results in rapid formation of OCs with features like multinucleation, TRAP+, demineralization, pit formation and collagen degradation.
- The rapid formation of OCs using H9 is due to increased gene expression and nuclear translocation of NFATc1 already in unstimulated H9. This could be especially valuable in initial larger screening experiments but also later in the scientific process i.e. to study osteoclastogenesis.

6.4 Paper IV

Aim

The aim was to study the role of TRAP 5b in OC differentiation. For this, we used a previously characterized TRAP 5b inhibitor, named 5-phenylnicotinic acid (5PNA)[251] that was shown to prevent the activation of the active center of TRAP 5b, thus rending the enzyme inactive. We examined how the inhibitor affects the differentiation of monocytes under RANKL and IL-4/IL-13 conditions, mimicking physiological and inflammatory conditions, respectively.

Toxicology studies

In order to evaluate whether the inhibition of osteoclastogenesis was due to a toxic effect we measured the metabolic activity based on the release of intracellular ATP

of hPBMCs treated with increasing concentrations of 5PNA (0-400 μM) and we found out that 5PNA was not toxic up to 200 μM and didn't induce morphological effects on the cells.

Inhibition of TRAP 5b inhibits OC differentiation

We observed a decrease in the number of OCs, and the ones that did differentiate appeared to be smaller than in the controls. We used a structurally unrelated TRAP-inhibitor Au[bipyOMe][249] and could see a similar tendency although the effect was less pronounced (Figure 1A), suggesting a role for TRAP in OCs formation.

Inhibition of TRAP 5b affects the transcription of OC markers

We examined the expression of OC markers upon 5PNA treatment in RANKL-induced induced human and mouse monocytes .We noticed that NFATc1 was downregulated in both human and mouse in response to the treatment. CtsK was consequently decreased in both human and mouse as NFATc1 coordinates the expression of several OC markers. We measured RANK expression in human monocytes and it showed a tendency in reduction. This suggests that 5PNA or TRAP 5b could be affecting early stages of osteoclastogenesis, for example affecting RANKL fusion regulators, thus preventing the OCs to form.

TRAP 5b inhibition affects resorption

As was previously shown by others, IL-4/IL-13-induced monocytes were not able to resorb bone but only the RANKL-induced ones could degrade the collagen type. It has been previously reported that TRAP knockout mice have OCs in vivo and when inhibiting TRAP and despite the lower resorption measurements after 5PNA, some cells were able to differentiate into OCs and resorb bone. The number of differentiated OCs was less and the resorption was consequently lower. However, it also seemed to be more bone resorbed underneath osteoclasts treated with 5PNA. We would need to measure the resorbed area and CTX in order to substantiate this observation. One possible explanation could be that the OCs can't migrate as fast in the presence of 5PNA and remain anchored for longer time to the same resorption pit.

TRAP 5a and 5b OCs production are altered after 5PNA in RANKL-induced OCs

The 5PNA treatment decreased the secretion of TRAP 5b but did not affect TRAP 5a secretion. TRAP 5b production seems to be decreased while TRAP 5a production shows a decrease tendency levels in both lysate and medium. The observed difference was more pronounced in lysate, but no apparent difference was detected in trafficking of TRAP (Figure 3). However, the 5PNA treated cells did generate TRAP isoforms, albeit slower than the untreated cells. Therefore, we concluded that the TRAP-inhibition did not disallow OCs differentiation, but reduced the efficiency of the process.

5PNA affects fusion of RANKL-induced but not in IL4/IL-13 – induced in hPBMCs and mBMMs

To examine whether TRAP 5b is involved in fusion and therefore could be a reason for the delayed differentiation of OCs and impaired overall resorption capacity, we stimulated PBMCs with IL-4 and IL-13 in order to form foreign body giant cells (FBGCs). These cells compared to the RANKL-induced produced equal amounts of TRAP 5a but significantly lower levels of TRAP 5b, and therefore, provide a control for TRAP 5b inhibition. It has been shown before that IL-4 is able to mediate fusion of monocytes by stimulating expression of DC-STAMP[131]. Moreover, it was also shown that IL-4 inhibits RANKL-stimulated osteoclast formation and large multinucleated TRAP positive cells are formed[134]. In PBMCs stimulated with IL-4 and IL-13, the fusion was unchanged with 5PNA treatment, furthermore the treatment showed a tendency in TRAP 5a secretion up to 50uM. This was different from the RANKL stimulated PBMCs, indicating that with the IL stimulation under 5PNA treatment the $CD14^+$ monocytes are still able to fuse. IL-4 was shown to block RANKL pathway, and they can use another set of molecules to fuse[134].

Conclusions

- With the TRAP activity inhibitor, we were able to show that TRAP activity was indeed promoting OC differentiation under RANKL stimulation.
- TRAP inhibitor did not seem to have an effect under interleukin stimulation.
- It seems that the TRAP inhibitor initially affects or delays the clustering of the monocytes in aggregates that eventually fuse into multinucleated OC on plastic. Also on bone, it looked like there are more monocytes remaining that did not form multinucleated OCs.

7 CONCLUDING REMARKS

1) The development of a novel TRAP 5a/5b ELISA allowed us to study the expression and secretion of the isoforms during OCs development, and show that OCs also secrete TRAP 5a under RANKL-induced conditions. Moreover, both isoforms were found to correlate to bone resorption marker, CTX-I, indicating that TRAP 5a levels can be used to study bone remodeling.

2) We studied the expression of TRAP 5a and 5b under inflammatory conditions, in patients with RA and OA and found out that the ratio 5b/5a determines the phosphorylated status of OPN, which contributes to a vicious cycle of inflammation, recruiting inflammatory cells, and leading to cartilage degradation and bone destruction.

4) Paper III was a methodological paper, were we showed that the RAW264.7 cells be used for screening purposes as they can form TRAP positive cells, acidify and resorb bovine cortical bone. The H9 clone form TRAP positive cells quicker than the parental RAW 264.7, possible because this clone is in a more differentiated stage, and allow to more stable results.

3) We have studied the biological role of TRAP in differentiating OCs, and we hypothesized that TRAP promotes RANKL-induced osteoclast differentiation in vitro, and is necessary for a normal resorption. However, it did not affect the formation of FBGCs.

8 FUTURE PERSPECTIVE AND CLINICAL SIGNIFICANCE

Bone remodeling is essential for a healthy skeleton, and imbalances in the resorption or formation of bone are known causes of several skeletal diseases. Knowing more about how osteoclastogenesis is regulated is essential for being able to propose new therapies. In the mark of this, the double TRAP 5a/TRAP 5b ELISA gives the possibility to redo everything that has been done before. We could better understand the role of TRAP 5a and TRAP 5b in different diseases, and possible give a better comprehension of the overall function of TRAP, as it has been probably masked by the fact that it was impossible to measure the protein content of it before, a redox status were affecting the overall TRAP activity.

TRAP 5b/5a ratio was shown to correlate with the phosphorylation status of OPN, but it could also correlate with several other prognosis markers in different bone diseases, Local supplementation of TRAP 5b in clinical trials could be a possibility. It seems interesting to evaluate 5a/5b ratios since it seems to be more sensitive for evaluation of the bone remodeling status for diagnostic purposes[264]. We show that lower levels of TRAP 5b/TRAP 5a correlated with a higher phosphorylated statues that could contribute to the inflammation cycle.

The identification of clones with different OC related makers from the RAW 264.7 cell line, that show different capabilities to form TRAP positive cells, indicates that this heterogenetic population can be sorted using these markers, so the high variability in the RAW 264.7 could be solved by sorting the cells based on NFATc1 and CtsK expression previous treatment with RANKL. These findings could be also applied in human monocytes in order to gather a uniform population of cells. It would be interesting to use the H9 in a high throughput system, searching for new OC inhibitors, as it represents a more stable and faster system than the RAW 264.7 parental. Resolving monocyte heterogeneity and understanding how their functional identity is determined holds great promise for therapeutic immune modulation

TRAP has been shown to be a real hallmark in osteoclastogenesis. *In vivo* studies are necessary to further understand how 5PNA affects bone remodeling. Study if treatment with TRAP 5b could rescue the phenotype in the $ACP5^{-/-}$ mice, could be a good strategy.

Lastly, although it was not studied in this thesis, but to understand the expression of TRAP in other bone cells seems necessary to comprehend the whole picture of TRAP in the bone remodeling unit. The role of TRAP in different bone cells, different from OCs seems, like the OBs an interesting subject for research behind the goal of understanding bone remodeling and improve therapy for several bone diseases.

The functional significance of a heterogeneous OC population still remains to be elucidated, and is an important goal to understand how a physiological or a pathological state could modulate OCs differentiation and their functions e.g. resorption[9,265]. The implications of site-specific OCs may be significant, especially when considering novel treatment options to inhibit resorption.

9 ACKNOWLEDGEMENTS

The work presented in this thesis was financially supported by grants from the **Swedish Research Council** and the **Marie Curie Euroclast Consortium**.

There are many people I owe my thanks to and I know it would not have been possible to finish this thesis without all of them. I feel extremely lucky to have met all these incredible people and would like to tell you that you have made this PhD journey a blast!

First and foremost, I would like to thank my main supervisor Pernilla Lång and my co-supervisors Göran Andersson and Tuomas Näreoja. **Pernilla**, thanks for having the patience to teach me so many things in the lab and for the interesting and fun scientific discussions. Thanks for helping me develop as a researcher, for supporting and encouraging me to find focus every time it was lost, I admire your love for science and I can not say how much I learned from you. **Göran**, thank you for giving me the opportunity to be part of your group, for continuously inspiring me with your enthusiasm for science and bright ideas and for the fun we had during the meetings around Europe. **Tuomas**, thanks for being my third supervisor, for encouraging me to try new things and teaching me so much not only about science but about how to deal with the scientific community. I had also a lot of fun arranging the world in our discussions.

To the members of my group, a big thank you to **Maria Norgård**, **Barbro Ek-Rylander** and **Sara Windahl** for all the good meetings and feedback. To the ex members of my group, **Christina Patlaka** and **Anja Reithmeier**, I do not know where I would be without you. Both of you supported me when I needed it the most, mentored me though scientific advice every time I asked for it and you help me to establish in Stockholm. However, what I value the most it is your friendship and your warm when I needed it the most. And of course the beers shared! **Anh N Tran,** I met you before I started this journey at the very first Euroclast meeting (through Skype), and our friendship has only gone bigger since then! You are a great scientist, with a bright mind and you have rendered yourself very important in my life lately! **Suchita Desai,** I enjoyed working with you during your master thesis, and I was happy to see that you were starting your PhD with Tuomas. I have had a lot of fun sharing time with you!

To my office colleagues/friends and former coworkers, **Magali Merrien** you and **Teresa Seestern** were the first people I started to hang out with, Teresa left, but we still talk here and then, and Magali has always been there for me, you are a very special person with a big heart and a great personality! **Joman Javadi**, **Zurab Bzhalava**, **Sara Arroyo**, **Mihaela Zabulica**, **Rim Jawad**, **Martin Lord, Arun Selva**, **Antje Zickler**, **Raghu Chittoor**, **Ashish Singh**, and all the students that

have been part of the division, I would have to write 10 pages to say how much fun I had all these years and how lucky I have been to share time with you. Special thank you also to **Agata Wasik** for the advices and for always being there when was needed. To **Mia Bjerke**, because without you I would be still figuring out how to send the application and also for always sharing your smile with all of us. To the rest of the division and to all PIs and seniors, for they constructive criticism at divisional seminars.

To all Euroclast partners, this got to an end but the journey was worth it, I enjoyed all the discusions about osteoclasts, about our projects, about our PhD, about beers, whiskies, and food! **Giuliana Ascone**, **Sara Sprangers**, **David Massa**, **Sandra Segeletz**, **Henrik Lövfall**, **Vikté Lionikaité**, **Yixuan Cao, Arjen Gebraad** and an extra thank you to **Emma McDermott** and **Miep Helfrich** for the beautiful pictures! And to all the supervisors, thanks for your input, sharing your experience with us and for the osteoclast discussions.

To all the friends I got in Stockholm, especially the ones imported from Valencia, **Patricia Gonzalez** and **Lorena Galan,** gracias por estar ahi siempre, por los brunchs y por vuestra amistad! To my hometown friends, especially **Estefania**, **Cristina**, **Sina** and **Aitor**, per fer-me vore que la distancia no era tan gran. And **Andrea,** per sempre tindre temps. And I can't forget all the **Cartaginenses**.

To my parents, **Teresa** and **Rafel**, for always supporting and encouraging me to reach out as far as I wanted and could. To my brother **Jaume,** perque et trobe molt a faltar i tindre't a prop! To the rest of my family for always being interested in knowing about my research, especially thanks to my aunt **Victoria** for looking after my demands! To my nephews, **Mateu**, **Jordi**, and also tomy son **Roger** because you all are cute and necessary, and life is better with you.

Finally, I consider myself the luckiest person to have met you, **Pep**, my partner, my friend and my love for your endless support, motivation and for always being there, thank you for waiting for me. You have no idea how comforting was to know that whenever I felt down you would lighten me up.

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