DEPARTMENT OF PATHOLOGY - INSTITUTE OF CLINICAL MEDICIN - UNIVERSITY OF OSLO AND
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Experimental studies on bone with focus on tartrate-resistant acid phosphatase and bone remodeling

PhD thesis

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Forget the great human heart and brain, the eyes that see sweeping vistas, the lips that declare "I am". The real thing standing between us and the primordial ooze is the human skeleton. Built of 206 bones, the skeleton is a living cathedral of ivory vaults, ribs and buttresses – a structure at once light and strong, flexible and firm.

Angier, N.
New York Times November 1994

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NORSK SAMMENDRAG

Beinskjørhet (osteoporose) er en forstyrrelse i skjelettstoffskiftet som gir økt beintap, redusert beinoppbygning og dermed mindre beinvev. Dette øker risikoen for beinbrudd, og hvert år pådrar over 25 000 nordmenn seg såkalte lavenergibrudd – som hoftebrudd, underarmsbrudd og brudd i ryggen forårsaket av et traume som hos beinfriske ikke ville medført skade. Folkehelseinstituttet har estimert at hoftebrudd alene koster samfunnet ca. 2 millarder NOK årlig og for den enkelte kan et slikt brudd være fatalt; ca 25 % av eldre med lårhalsbrudd dør i løpet av det første året etter bruddet. Vi vet mye om hvilke faktorer som øker risikoen for osteoporose, imidlertid er det fremdeles uavklarte molekylære mekanismer bak denne vanlige lidelsen. Det er derfor både i den enkelte pasients og samfunnets interesse at kunnskapen om de molekylære mekanismene ved osteoporose økes, slik at vi kan utvikle bedre behandling og ikke minst kunne forutse og forebygge osteoporose og lavenergibrudd.

I det første arbeidet i avhandlingen ”Experimental studies on bone with focus on tartrate-resistant acid phosphatase and bone remodeling” har cand.med. Lene B. Solberg og medarbeidere studert sammenhengen mellom osteoporose og bruddtilheling hos rotte. Ved å måle beintetthet og mekaniske egenskaper til bruddområdet under tilheling fant gruppen ingen forskjeller mellom osteoporotiske og normale dyr, og konkluderer med at brudd hos osteoporotiske rotter gror som normalt. I de to påfølgende arbeidene har Solberg og medarbeidere studert enzymet tartrate-resistant acid phosphatase (TRAP) som blant annet produseres og skilles ut fra beinnedbrytende celler (osteoklaster). En beinspesifikk type av TRAP (TRAP 5b) kan måles i serum og flere studier har vist at mengden TRAP 5b i serum er direkte proporsjonal med osteoklastantallet og har prognostisk og diagnostisk verdi i kartlegging av osteoporose. Imidlertid er TRAPs direkte rolle i beinstoffskiftet ukjent. Solberg og medarbeidere har brukt rotter med osteoporose og D-vitaminmangelsykdom samt unge rotter i vekst til å studere omsetningen av TRAP i beinvev. Ved hjelp av ulike lys- og elektronmikroskopiske teknikker fant gruppen økt nivå av TRAP i flere typer beinceller (osteoblaster og osteocytter) hos osteoporotiske rotter. Gruppen fant TRAP sammen med en faktor som regulerer beinnedbrytningen (RANKL) og som skilles ut fra osteoblaster og ostecytter. Disse nye funnene taler for at TRAP kan ha flere oppgaver i bein, og gruppen foreslår at TRAP har en rolle i reguleringen av beinnedbrytningen og/eller er et signalmolekyl i koblingen mellom beinnedbrytning og beinoppbygging.

ACKNOWLEDGEMENTS

The studies included in this thesis have been carried out at the Department of Pathology, Oslo University Hospital Rikshospitalet in the Laboratory of Electron Microscopy. I started my scientific career as a medical student at the Medical Student Research Program (MSR) from 2003 to 2006, financed by the Research Council of Norway and the University of Oslo. After graduating from the Faculty of Medicine, University of Oslo in 2007, I had my internship at Molde Hospital where I also have worked as a resident. In April 2010 I got a personal grant from the Norwegian Sout-Estern Regional Health Authority (Helse Sør-Øst) which financed the project until September 2013. I would like to express my gratitude towards the Department of Pathology, the MSR and the University of Oslo and for the funding provided.

Several people, including family, friends and colleagues, have contributed to my work over the last years and I am most grateful for your valuable inputs, comments and criticism. However, the following people deserve a special recognition.

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My co-supervisors *Sverre-Henning Brorson* and *Lars Nordsletten* have played important roles in the project. Sverre, your enthusiasm and great knowledge in the field of electron microscopy and immunogold techniques have been crucial for my projects. Your “never ending” new ideas and your ability to transform them into research initiated my second project. Lars, your networking ability and true interest in research makes you a great capacity in the field of orthopedic research. I am glad you guided me (and Gunhild) to Finn at the start of this journey. I am also grateful that you introduced me to the stimulating and dynamic orthopedic department at Ullevål.

My co-authors: *Gunhild Stordalen, Sigbjørn Dimmen, Jan-Erik Madsen, Espen S. Bækkevold, Espen Stang* and *Göran Andersson* who I have had the pleasure of working with also deserve a special thank-you. Gunhild for being my partner and friend during the years at the research curriculum; Sigbjørn and Jan-Erik for teaching Gunhild and me surgical procedures on small animals and for being exemplary orthopedic colleagues; Espen B for helping me with the in situ hybridization; Espen S for providing exceptional intracellular immunogold staining on bone tissue and last, but not least - Göran for sharing your knowledge on TRAP with me, and for never being tired of my questions and suggestions and for inviting me to use the very nice facilities you dispose at the Karolinska Institutet.

Aileen Murdoch-Larsen, Linda T. Dorg and *Linda I. Solfjell* also deserve my thankfulness: Aileen for great technical assistance and specimen preparation during the first experimental period, Linda S for teaching me molecular biology and Linda D for being my co-worker and friend for the last 3 years – without you and your knowledge in all the technical procedures involved in this thesis I am not sure it would have been a thesis... *Merete Helgesen* and *Jon Halvor Lunde* at the EM lab definitively made the days at the Institute of Pathology more fun! *Vibeke Bertelsen*, with whom I shared the office - your sharing of experiences as a former phd student, researcher and most important as a mother of two has been most valuable to me.

My patient family deserves more than a big thank-you! *Anders Nordby*, my closest friend and partner in life; you have always supported me in my research regardless of the late working nights and weekends at the lab making you a single dad for Eir, our little daughter. Without you I wouldn't have had the most important thing in life – my own family – you and Eir mean the world to me.

Lene Bergendal Solberg
Molde February 2014

ABBREVIATIONS

Symbols for genes and their associated proteins in humans are given according to the Hugo Gene Nomenclature Committee (HGNC) July 2013 [1].

1,25D	Calcitriol or active vitamin D
ACP5	Acid phosphatase 5, tartrate resistant
ACPases	Acidic phosphatases
ALP	Alkaline phosphatase
B.Pm	Bone perimeter
BAD	BCL2-associated death promoter
BMD	Bone mineral density
BMI	Body mass index
BMPs	Bone morphogenic proteins
BMU	Bone multicellular unit
CART	Cocaine- and amphetamine-regulated transcript
Cd^{2+}	Cadmium
CO^{3-}	Carbonate
COL1	Collagen type I
CSF1	Colony stimulating factor 1 (previous M-CSF)
CSF1R	Colony stimulating factor 1 receptor (previous c-fms)
CTSK	Cathepsin K
CTX-I	C-terminal telopeptides from collagen type I
DEXA/DXA	Dual-energy X-ray absorbtiometry
DKK1	Dickkopf WNT signaling pathway inhibitor 1
DMP1	Dentin matrix acidic phosphoprotein 1
ECM	Extracellular matrix
EFNB2	Ephrin-B2
ELF97	Enzyme-Labeled Fluorescence® 97
EPHB4	EPH receptor B4
ER	Estrogen receptor
FAS	Fas cell surface death receptor
FASLG	Fas ligand (TNF superfamily, member 6)
FGFs	Fibroblast growth factors
FRAX	WHO fracture risk assessment tool
FSD	Functional secretory domain (in osteoclasts)
GH	Growth hormon
HAP	Hydroxyapaptite
HMBG1	High mobility group box 1
HZ	Hypertrophic zone (in the epiphyseal growth plate)
IBSP	Integrin binding sialoprotein
IGFI/II	Insulin-like growth factor 1 or 2
IHH	Indian hedgehog

IL6	Interleukin 6
ITAM	Immunoreceptor tyrosin-based activation motif
kDa	kilo dalton
LAMP1	Lysosomal-associated membrane protein 1
LCD	Low calcium diet
LRPs	Low density lipoprotein receptor-related proteins
MDGF	Macrophage derived growth factor
MEPE	Matrix extracellular phosphoglycoprotein
Mg^{2+}	Magnesium
MMPs	Matrix metalloproteinases
MPC	Mesenchymal progenitor cell
mRNA	Messenger RNA
mTRAP	Monomeric tartrate-resistant acid phosphatase
N	Newton
N.Oc	Number of osteoclast
NCPs	Non-collagenous proteins
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin dependent 1
NO	Nitric oxide
NOK	Norwegian kroner
NS	Not significant
NSAIDs	Non-steroidal anti-inflammatory drugs
Ob	Osteoblast
Oc	Osteoclast
OCN/BGLAP	Osteocalcin/Bone gamma-carboxyglutamate (gla) protein
ON/SPARC	Osteonectin/Secreted protein, acidic, cysteine-rich
OPG/ TNFRSF11B	Osteoprotegerin/Tumor necrosis factor receptor superfamily, member 11b
OPN/SPP1	Osteopontin/Secreted phosphoprotein 1
OSX/SP7	Osterix/Sp7 transcription factor
Ot	Osteocyte
OVX	Ovariectomy
Ovx-D	Ovariectomy and vitamin D deficiency
PAP	Purple acid phosphatase
PDGF	Platelet derived growth factor
PDPN	Podoplanin (E11/gp38)
PGE2	Prostaglandin E2
PHEX	Phosphate-regulating gene with homologies to endopeptidases on the X chromosome
PPR1	PTH/PTHrP receptor 1
PS	Primary spongiosa
PTH	Parathyroid hormon
PTHrP	Parathyroid hormone related peptide
PZ	Proliferative zone (in the epiphyseal growth plate)
QCT	Quantitative computed tomography
Rad/s	Radians per second = displacement
RANK/ TNFRSF11A	Receptor activator of NFKB/Tumor necrosis factor receptor superfamily, member 11a, NFKB activator
RANKL/ TNFSF11	Receptor activator of NFKB ligand/Tumor necrosis factor (ligand) superfamily, member 11

RB	Ruffle border (in osteoclasts)
RCT	Randomized control trial
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2
RXR	Retinoid X receptor
RZ	Resting zone (in the epiphyseal growth plate)
SNS	Sympathetic nervous system
SOC	Secondary ossification center
SOST	Sclerostin
SOX9	SRY (sex determining region Y)-box 9
Sr^{2+}	Strontium
SZ	Sealing zone (between osteoclasts and ECM)
T.Ar	Tissue area
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alfa
TRAP	Tartrate-resistant acid phosphatase
Tv	Transcytotic vesicle
UVB	Ultraviolet B
VDR	Vitamin D (1,25- dihydroxyvitamin D3) receptor
VDRE	Vitamin D responsive elements
VEGF	Vascular endothelial growth factor
VPS33A	Vacuolar protein sorting 33 homolog A (S. cerevisiae)
WHO	World Health Organization
WNT	Wingless-type MMTV integration site family

LIST OF PAPERS

The following papers are included in this thesis and will be referred to by their Roman numerals in the text.

I. Melhus G*, Solberg LB*, Dimmen S, Madsen JE, Nordsletten L, Reinholt FP. Experimental osteoporosis induced by ovariectomy and vitamin D deficient diet does not markedly affect fracture healing in rats
Acta Orthop 2007 Jun;78(3):393-403
*The authors contributed equally to the study

II. Solberg LB, Brorson SH, Stordalen GA, Bækkevold E, Andersson G, Reinholt FP. Increased tartrate-resistant acid phosphatase (TRAP) expression in osteoblasts and osteocytes in experimental osteoporosis in rats
Submitted October 2013

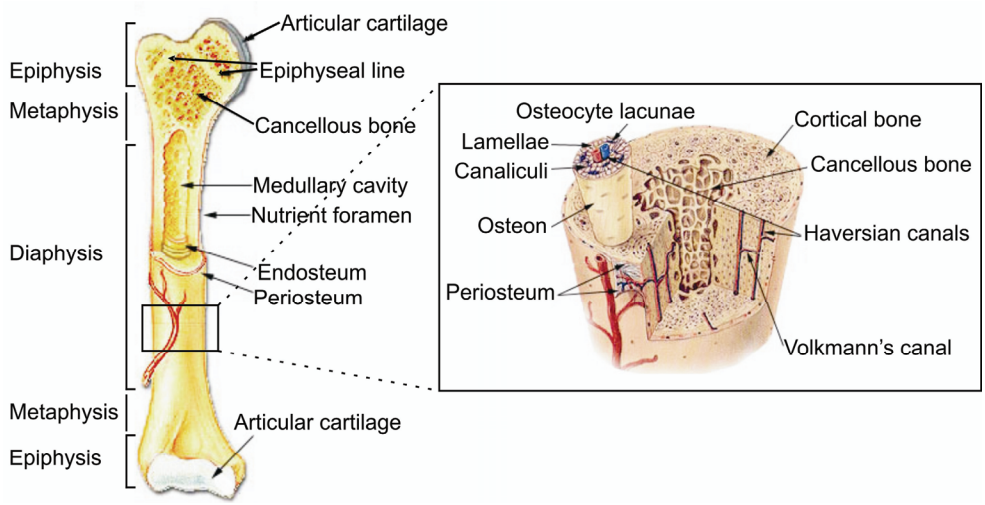
III. Solberg LB, Stang E, Brorson SH, Andersson G, Reinholt FP. Co-localization of tartrate-resistant acid phosphatase (TRAP) and receptor activator of NFKB ligand (RANKL) in lysosomal associated membrane protein 1 (LAMP1) positive vesicles in osteoblasts and osteocytes in rats
Manuscript November 2013

1 INTRODUCTION

The bony skeleton is an organ constantly being changed and rebuilt in order to maintain its structural and metabolic functions. It provides body support critical for locomotion, respiration and protection of the internal organs as well as houses the bone marrow and sharing cells and local regulatory factors with the hematopoietic system. The bone tissue components include the mineralized and un-mineralized connective tissue matrix, bone cells and water and serve as an ion reservoir, crucial for the calcium and phosphate homeostasis in blood. The importance of studying skeletal physiology and pathology is illustrated by the incidence of bone diseases affecting the various skeletal functions such as the control and arrest of skeletal longitudinal growth, bone mineralization and control of bone mass manifested i.e. as dwarfism, rickets and osteoporosis as well as some less prevalent diseases such as osteogenesis imperfecta and sclerostosis.

1.1 Bone biology

Newborn and infants have between 300-350 bones which fuse at different time-points during skeletal maturation and growth to the 206 bones making up the adult skeleton. The *axial skeleton* comprises the bones in the midline (skull bones, hyoid bone, vertebrae, sternum and ribs) while the *appendicular skeleton* includes the extremities, scapulas, clavicles and pelvis. The individual bones can be further classified depending on their gross appearance and architectural structure; most bones of the appendicular skeleton (such as femur and tibia) are *long bones* (Fig. 1), whereas *flat bones* are found in the skull, mandible, scapula and pelvis. *Cortical bone* makes up the dense outer shell of all bones and the diaphysis of long bones providing mechanical strength and protection, while *cancellous bone* is found in the center of flat bones and underneath the cortical shell as well as in the metaphysis of long bones.



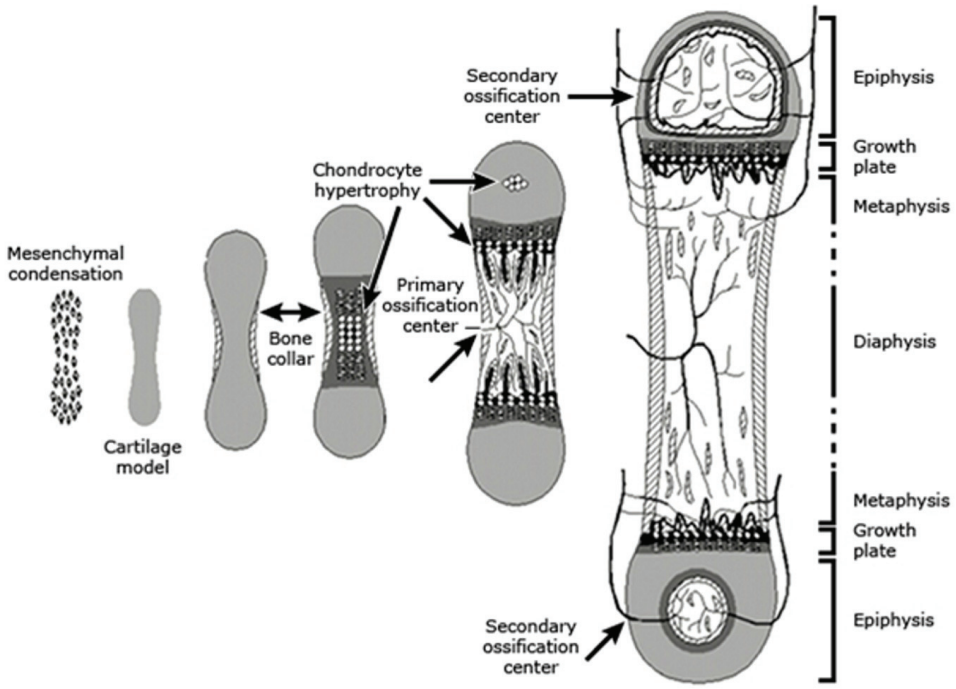
Modified images from Wikimedia Commons, the free media repository;
http://commons.wikimedia.org/wiki/File:Illu_long_bone.jpg;
http://commons.wikimedia.org/wiki/File:Illu_compact_spongy_bone.jpg

Fig. 1 The anatomy of long bones The *epiphyses*, *metaphyses* and *diaphysis* in humerus with the insert showing details of the cortical and cancellous bone. Cortical bone, comprises 80% of the skeletal weight, is compact with tightly packet lamellae or *osteons* characterized by collagen fibers oriented axial to the applied mechanical force wrapped around a central (*Haversian*) canal which contains blood and lymphatic vessels and nerves. The vessels enter bone from the periosteum or the bone marrow through *Volkmann's canals* running perpendicular to the Haversian canals. C*ancellous bone* has a "spongy" appearance with thin bony plates (trabeculae) forming a complex network hosting the bone marrow. A high surface-to-volume ratio and close relation to the circulation enable cancellous bone to play a key role in the circulating calcium and phosphate homeostasis. The *endosteum* is a thin delicate membrane of osteogenic cells lining the bone adjacent to the bone marrow, while the outer surface is covered with the *periosteum* (except for where it's coated with articular cartilage) serving as attachment for ligaments and giving rise to the osteogenic cells.

1.1.1 Bone development and growth

The embryonic bone tissue develops from condensations of mesenchymal cells and is formed either directly by differentiation of the mesenchymal cells into bone-forming osteoblasts (*intramembranous ossification)*; or in a multi-level process designated *endochondral ossification* where cartilaginous structures are built, resorbed and replaced by bone (Fig. 2). Intramembranous bone formation occurs in the skull, part of the facial bones and clavicles in addition to the periosteal column of long bones, whereas endochondral bone formation occurs in the rest of the axial and appendicular skeleton. The embryonic bone enlarges by appositional growth, while the *epiphyseal growth plate* enables continued elongation of long

bones through chondrocyte proliferation and hypertrophy as long as the growth plate exists. At the time of puberty, sex hormone levels increase leading to a phase of accelerated bone growth followed by closure of the epiphyseal growth plates. Although longitudinal growth of long bones ceases after puberty, bone formation continues in an appositional manner via intramembranous bone formation in the diaphysis increasing the transverse diameters of the long bones.



From: Diseases of bone and mineral metabolism, Singer F (Ed). Copyright © 2008 WWW.ENDOTEXT.ORG Reproduced with permission from Dr. Roland Baron.

Fig. 2 Endochondral ossification The mesenchymal cells condensate and differentiate into chondrocytes producing a cartilage template. The cells at the border form the *perichondrium* which transforms into the periosteum and the bone collars which become the future cortical bone. The chondrocytes in the center hypertrophy and mineralize their surroundings, and attract capillaries, osteoblasts and chondroclasts/osteoclasts to the mineralized cartilage before they die. The invading cells form the *primary ossification center* where osteoblasts synthesize immature (woven) bone upon the cartilage remnants becoming the future cancellous bone. As the bone grows, the central part is gradually resorbed and populated with myeloid stromal cells establishing the main site of the hematopoiesis in post-natal life. *Secondary ossification centers* (SOCs) develop (usually after birth) in the same manner as the primary centers. In long bones, SOCs are formed in the epiphysis leaving only a cartilaginous growth plate between the primary and secondary ossification centers.

1.1.2 The epiphyseal growth plate

When secondary ossification (Fig. 2) is complete, the cartilage model is totally replaced by bone except for in two areas; a region of articular cartilage remains at the distal end of the epiphysis as well as a transverse disc of cartilage between the epiphysis and the diaphysis called the *epiphyseal growth plate*. The growth plate contains chondrocytes at different stages of development, and proliferation and differentiation of these cells provide elongation of the long bones (Fig. 3).

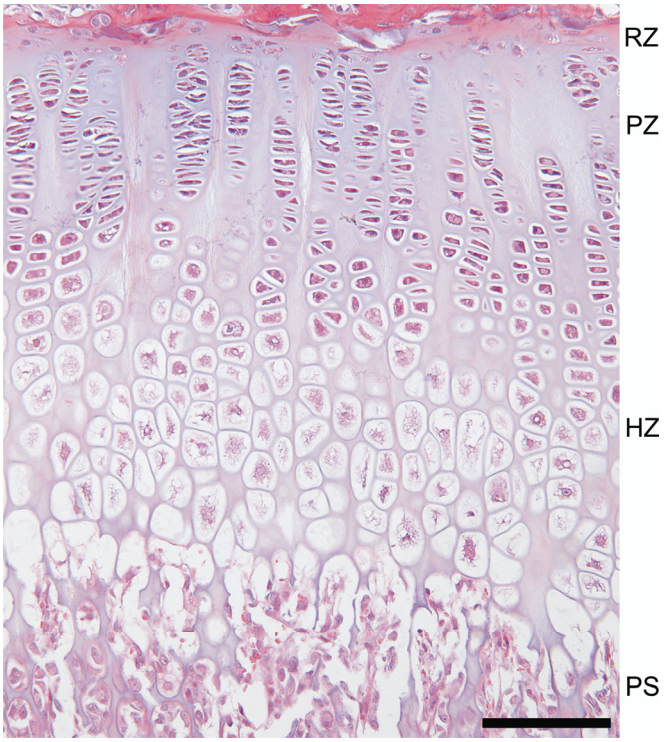


Fig. 3 The epiphyseal growth plate Light microscopic image from a 10 weeks old normal male rat. The growth plate can be divided into 3 zones; the resting zone (RZ), the proliferative zone (PZ) and the hypertrophic zone (HZ) next to the primary spongiosa (PS) - the initial trabecular network remodeled into cancellous or cortical bone. The cartilaginous cells proliferate as long as the growth plate exists. However, the epiphyseal disc does not increase in thickness as the cartilage matures and mineralizes because the cartilage is resorbed and replaced by primary spongiosa at the same speed in a tightly controlled manner. Scale bar 100µm

1.1.3 Regulation of chondrogenesis

The chondrogenesis in both embryonic and post-natal endochondral bone formation is strictly regulated to ensure normal longitudinal bone growth. The differentiation of condensated mesenchymal cells into chondrocytes is crucial for the development of the cartilaginous model. The differentiation is driven by cell-extracellular matrix (ECM) and cell-cell adhesion interactions mediated by hormonal and local factors controlling the key transcription factors SOX9 and RUNX2. SOX9 is one of the earliest markers of the chondrocyte lineage and crucial for the differentiation of condensated mesenchymal cells into chondrocytes, while RUNX2 is the main inducer of hypertrophic differentiation and is expressed by prehypertrophic and hypertrophic chondrocytes. Members of the WNT family regulate chondrocyte differentiation from mesenchymal precursor cells (MPC) via chondrocytes to hypertrophic chondrocytes in different ways; e.g. WNT5a and 5b (non-canonical pathways) stimulate differentiation from MPC to mature chondrocytes. Loss of β-catenin activity is required in mesenchymal cells to permit chondrocyte differentiations, but inhibits further differentiation into hypertrophic chondrocytes (reviewed in [2-4]). The Indian hedgehog protein (IHH) is expressed in prehypertrophic chondrocytes and is required for normal chondrocyte proliferation as well as coupling the chondrocyte hypertrophy with osteoblastogenesis by signaling to the adjacent perichondrium to induce osteoblast differentiation. IHH also induces PTH (parathyroid hormone)-related peptide (PTHrP) in articular cartilage and periarticular cells (chondrocytes in the distal ends of the condensation). The PTH/PTHrP receptor (PPR1) is expressed at much higher levels in the proliferating and prehypertrophic chondrocytes, and activation of PPR1 prevents the onset of hypertrophic differentiation and thus the differentiation of IHH expressing cells. This forms a negative feedback-loop controlling the proliferating chondrocytes (reviewed in [2, 3]). IHH expression is promoted by bone morphogenic proteins (BMPs) and stimulates chondrocyte proliferation, while the fibroblast growth factors (FGFs) inhibit proliferation. Circulating hormones also play a crucial role in controlling the growth and inhibitory factors secreted by the chondrocytes during the different stages of differentiation (reviewed in [5]); e.g. growth hormone (GH) from the pituitary gland stimulates the secretion of insulin-like growth factor 1 (IGF-1) necessary for both chondrocyte proliferation and hypertrophy; thyroid hormone-induced hypertrophy appears to be mediated through the WNT/β-catenin (canonical) pathway. In addition to limiting the proliferation rate, the hypertrophic chondrocytes regulate cartilage mineralization by releasing matrix vesicles to the surrounding ECM [6, 7] providing the

nucleation site for mineralization. The hypertrophic chondrocytes also regulate the invasion of vessel-forming cells, chondroclasts/osteoclasts as well as osteoblasts to the mineralized cartilage by expressing vascular endothelial growth factor (VEGF) promoting the vascular invasion, high-mobility group box 1 protein (HMGB1) acting on endothelial cells, osteoblasts and osteoclasts, as well as receptor activator of NFKB ligand (RANKL) [8] which is essential for chondroclast/osteoclast differentiation (reviewed in [5]). Estrogen regulates the closure of the epiphyseal growth plate and the skeleton maturation in human males as well as females, and estrogen receptor (ERα) expressed by the growth plate chondrocytes is a likely mediator of these effects [9-11].

1.2 Composition of bone tissue

The bone tissue consists of mineralized and non-mineralized matrix components as well as the bone cells. According to volume, the bone mineral hydroxyapatite (HAP) constitutes approximately 50% of the ECM, the rest being a mixture of collagen fibers, NCPs, proteoglycans, lipids and water where the exact composition varies dependent upon age, anatomic location, diet and general health status of the individual.

1.2.1 Bone cells

There are 3 different cells types in bone originated from 2 different cell lineages; mesenchymal cells give rise to the osteoblast lineage, which includes lining cells and osteocytes, in addition to adipocytes, myocytes and chondrocytes. The other cell line is the hematopoietic monocyte-macrophage lineage in the bone marrow which gives rise to the cartilage and bone macrophages known as chondroclasts/osteoclasts.

The osteoblast is the bone forming cell, columnar in shape with a single, non-centric nucleus in its mature, active stage. Osteoblasts synthesize the bone matrix (*osteoid*) which comprises mostly collagen type I (COL1) as well as some minor collagens and proteoglycans, in addition to many of the NCPs such as integrin binding sialoprotein (IBSP), osteopontin (OPN), osteocalcin (OCN), osteonectin (ON) and the membrane-bound enzyme alkaline phosphatase (ALP), known to be an osteoblast marker. Osteoblasts originate from mesenchymal progenitor cells in the periosteum and bone marrow as well as in perivascularly. RUNX2 and Osterix (OSX) are two key transcription factors in osteoblast differentiation and facilitate the regulation of a variety of the different osteoblast-specific genes ([12]). RUNX2 is one of the earliest markers of the osteoblast lineage and acts directly on the promoter for

OSX. Both Runx2 and Osx -/- mice completely lack osteoblasts demonstrating the essential role of these two transcription factors (reviewed in [13]). Numerous signaling pathways are involved in the differentiation, activation and inhibition of osteoblastic bone formation mediated through RUNX2 and OSX, among these are the BMPs, the WNTs, TGF-β, PTH, IGF-1, the FGFs and Notch (reviewed in [13-16]). In the end, 60-80% of the osteoblasts die by apoptosis. The remaining cells are either embedded in their own matrix during deposition of osteoid becoming *osteocytes* or they are converted into less active, flatten *lining cells* covering the bone surface.

The osteocyte is the most abundant bone cell and is located in small lacunar areas in the mineralized matrix. It is stellate shaped with long cytoplasmic processes radiating from the cell body through small canals (*canaliculi)* in the mineralized matrix. These cytoplasmic processes connect the osteocytes to each other, the vessels in the Haversian canals, the bone surface with lining cells and osteoblasts as well as the cells in the bone marrow. During the transformation from mature, bone forming osteoblast to matrix embedded osteocyte several genes are switch on and off; one of the earliest osteocyte markers is E11/gp38/Podoplanin (PDPN), which seems to facilitate the formation of the dendritic processes/canaliculi. ALP is reduced, while casein kinase II and OCN is elevated in the late osteoblast and also expressed by the osteocyte. The mature osteocyte expresses markers such as phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX), matrix extracellular phosphoglycoprotein (MEPE), dentin matrix acidic phosphoprotein 1 (DMP1) and fibroblast growth factor 23 (FGF23) known to regulate phosphate metabolism. When the osteocyte senses the fluid-flow stress in the surrounding gel-matrix the cell responds by regulation of e.g. nitric oxide (NO), prostaglandin E2 (PGE2) and sclerostin. Sclerostin is a protein exclusively produced by osteocytes. It inhibits the canonical WNT/β-catenin signaling pathway reducing osteoblast differentiation from mesenchymal precursors and subsequent bone formation (reviewed in [17, 18]). Recently it has also been clarified that the osteocytes are the main source of RANKL in adult bone [19, 20] which is the main supporter of the osteoclastogenesis (reviewed in [14]) (Fig. 4). Osteocytes are also able to remodel their own environment (*osteocytic osteolysis*) under certain conditions, such as lactation, probably through the same mechanisms as the osteoclasts and in response to urgent need for bone mineral [21].

The osteoclast is a multinucleated giant cell formed by fusion of mononuclear precursor cells of the monocyte-macrophage cell lineage derived from the circulation. The development of the osteoclastic phenotype is initially induced by the binding of colony

stimulating factor 1 (CSF1) to its receptor CSF1R which upregulates receptor activator of NFKB (RANK) on the cell surface (reviewed in [14]).

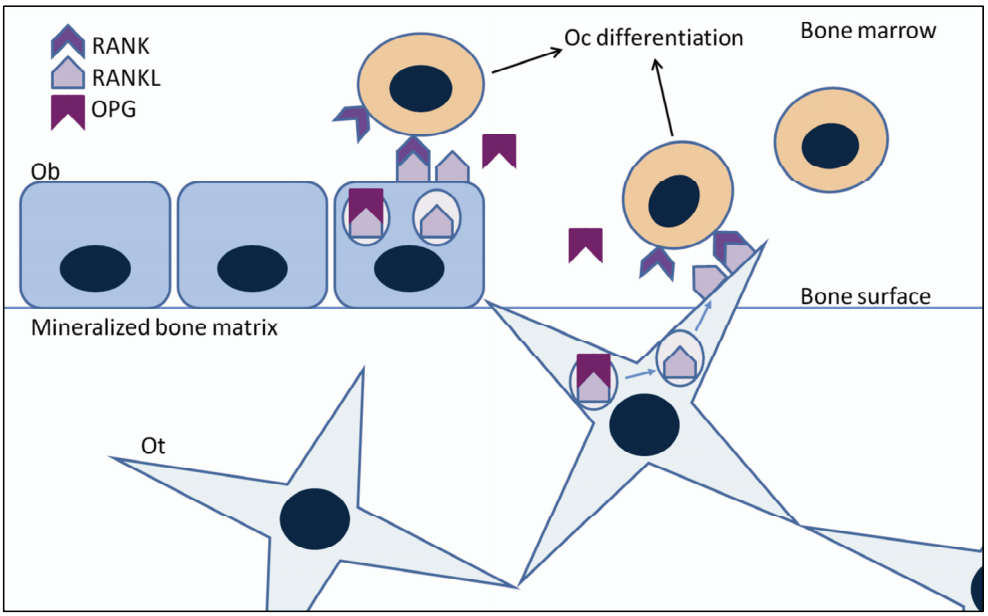


Fig. 4 The RANK-RANKL-OPG axis The figure shows how membrane-bound RANKL in osteoblasts (Ob) and osteocytes (Ot) binds to RANK at the surface membrane of mononuclear precursor cells stimulating osteoclast (Oc) differentiation and activation [22, 23]. OPG may act as a decoy receptor for RANKL in addition to be an intracellular traffic regulator for RANKL to secretory lysosomes in both osteoblasts and osteocytes [23, 24].

RANKL, expressed by osteoblasts and osteocytes in addition to hypertrophic chondrocytes, is essential for further differentiation into bone resorbing osteoclasts and loss of function mutations in RANK or RANKL causes severe osteopetrosis (a high bone mass disease) due to impaired osteoclast function [25, 26]. It is not fully clarified whether membrane-bound RANKL or soluble RANKL activates RANK, however, it seems like membrane-bound RANKL is the most efficient osteoclast activator [22], (reviewed in [27]), and together with co-stimulation by the immunoreceptor tyrosin-based activation motif (ITAM)-containing adaptors, this leads to activation of the transcription factor NFATC1. NFATC1 is crucial for the osteoclastic phenotype and regulates the osteoclast specific genes (i.e. cathepsin K (CTSK), tartrate-resistant acid phosphatase (TRAP), matrix metalloproteinase 9 (MMP9) and β3-integrin) (reviewed in [14, 18, 28]). Osteoprotegerin (OPG), which is produced by osteoblasts and osteocytes in addition to B cells, endothelial cells and vascular smooth muscle

cells, serves as a decoy-receptor for RANKL and by binding to RANKL inhibits the activation of RANK (reviewed in [28, 29]) (Fig. 4). Continued stimulation through CSF1R and RANK is required for osteoclast survival and thereby making the RANK-RANKL-OPG axis a suitable target for therapeutic agents aiming at diminishing bone resorption.

Prior to bone resorption, the maturing osteoclast must attach to the targeted mineralized matrix before the final differentiation occurs. The binding of osteoclastic vitronectin receptor ($\alpha_v\beta_3$-integrin) to e.g. OPN enriched at the bone surface [30, 31], seems to facilitate the further polarization and reorganization of the actin cytoskeleton forming an F-actin ring "sealing" the osteoclast to the surface (sealing zone, SZ). This isolates a membrane area that develops into the ruffled border (RB) (reviewed in [14, 32]). Simultaneously, a functional secretory domain (FSD) develops at the basolateral side allowing the phagocytosed bone constituents to be secreted into the circulation [33]. The SZ and the RB form a closed space, *the osteoclast resorption pit*, where acidification of the environment promotes bone resorption (Fig. 5). What stops the osteoclastic bone resorption is not fully clear, but after a few weeks the osteoclast undergoes apoptosis. Estrogen via TGF-β as well as soluble Fas ligand (FASLG) from the osteoblasts acting on the Fas receptor (FAS) in the osteoclasts membrane are known to decrease bone resorption by inducing osteoclast apoptosis. These mechanisms are further detailed in 1.3.

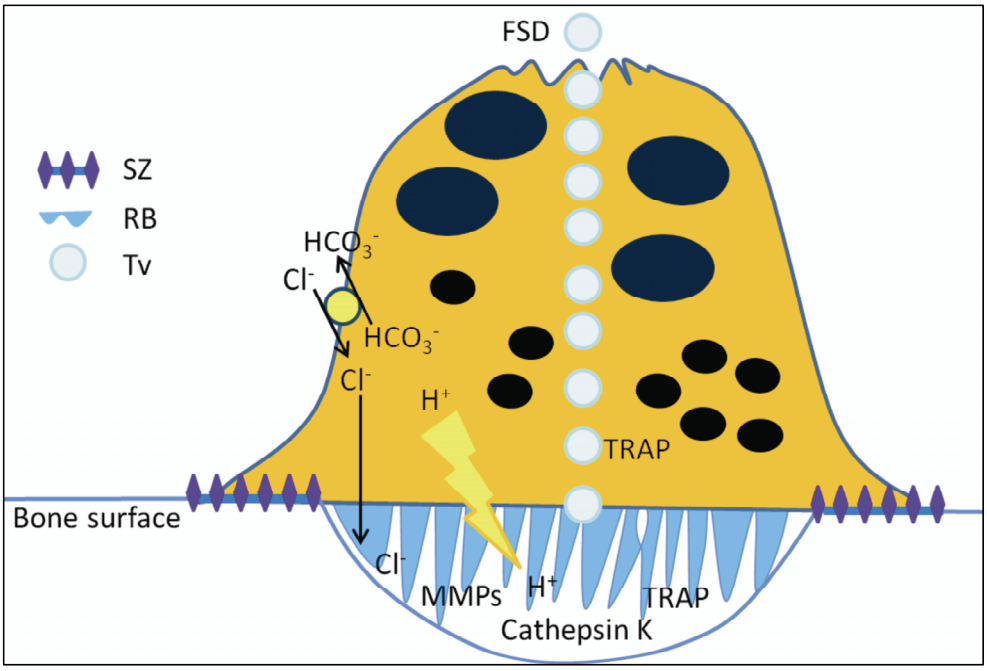


Fig. 5 The bone resorbing osteoclast (Oc) attaches to bone via $\alpha_v\beta_3$-OPN binding creating a sealing zone (SZ). An isolated membrane area develops into the ruffled border (RB) exposing a large surface area towards the bone. Bone resorption occurs at low pH promoting dissolution of bone mineral and degradation of collagen type I by acidic proteases such as cathepsin K and the MMPs. Collagen remnants, calcium (Ca^{2+}) and phosphate (PO_4^{3-}) as well as other molecules are endocytosed and secreted into the circulation via the functional secretory domain (FSD). TRAP may serve as a detachment-factor promoting osteoclast migration by dephosphorylation of e.g. OPN [34] as well as collaborate in the degradation of bone remnants in transcytotic vesicles (Tv) together with cathepsin K [35].

1.2.2 Extracellular bone matrix

In all bone types the ECM consist of collagen fibrils, NCPs, small amounts of phospholipids, serum proteins and water in addition to HAP. In compact bone collagen type I comprises over 90% of the organic matrix while the rest of the bone proteins being NCPs, however the ratio differs between the various bone types, with woven bone (newly formed bone) being the one with the highest NCPs content (reviewed in [36]).

Collagens make up the structural framework in bone and other connective tissues and represent a large family of proteins, accounting for nearly 30% of all human proteins. In bone, the collagens fibrils contribute to its fracture resistance and elastic properties, as well as playing an important role as a structural template for mineral deposition. Collagen type I is predominant in bone, while collagen type II is the main constituent in hyaline cartilage. Other

collagens are present as well, however in small amounts. Collagen I and II are rod-like structures derived from the aggregation of 3 polypeptide chains (in collagen type 1: 2 α1(I) chains and 1 α2(1) chain) fold into a triple helix molecule, where the polypeptide chains are characterized by a triplet repeat sequence (gly-x-y, where gly is glycin, x usually is proline and y often hydroxyproline). The collagen molecules assemble into fibrils stabilized by cross-links within and in-between the collagen fibrils. These bone-derived collagen cross-links (CTX) can be measured in the urine to monitor bone resorption.

Non-collagenous matrix proteins are a heterogeneous group of different proteins participating and controlling bone mineralization as well as being involved in bone and energy metabolism (e.g. OCN). Most NCPs are acidic proteins containing glutamic acid, aspartic acid, and phosphorylated serine/threonine residues. In addition, many of them have a high capacity for binding calcium ions and to HAP crystal surfaces [37]. The NCPs can be divided into subgroups according to their main components as proteoglycans, glycoproteins or γ-carboxylated (gla) proteins. Most of the NCPs are produced locally by the cells of the osteoblastic lineage, but approximately one-fourth are produced elsewhere, mainly in the liver. These exogenously derived proteins are transported in the circulation and suggested to bind to bone matrix because of their high affinity to HAP. Many are acidic serum proteins (such as albumin and α2-HS-glycoprotein) which may be of importance in bone mineralization or play a role in circulating calcium transport. The NCPs are suggested a role as biological modulators rather than exhibiting structural support. Their regulatory function of mineralization as well as crystal growth within osteoid is supported by knock-out/transgenic experimental studies reporting dramatic phenotypic abnormalities in the mineralization process (the function of each protein is reviewed in [36, 38]). However, their concerted role in the mineralization of bone is still elusive.

1.2.3 Matrix mineralization

Bone mineral is essential for the mechanical properties and integrity of the bone tissue and serves as the most important ion reservoir of the body. Bone mineral is present mainly as a complex of calcium and phosphate, quite close to naturally occurring geologic hydroxyapatite, $[Ca_{10}(PO_4)_6OH_2]$, but the crystals are smaller, less perfect and contain several impurities. The imperfect composition of the HAP crystals provides increased solubility and eases the release of ions into the circulation. However, as the bone matures the crystals increase in size and become more perfect. The HAP crystals are deposited between the

collagen fibrils and there are two essential events in the mineralization process of bone; the first is the nucleation of the crystals with a length axis, the other is the crystal growth. Matrix vesicles, mineralization foci, "crystal ghosts", calcospherulites, phosphoproteins-collagen fibrils and α2-HS-glycoprotein have been suggested candidates responsible for the initiation and controlling of the mineralization process, thus it is likely that they all, to some extent, play a role (reviewed in [38]). After the initial mineral deposition into the "holes" between the collagen fibrils, the crystals enlarge and mature by the aggregation of new ions enabling growth to occur in more than one direction in an exponential fashion. The NCPs act probably as controllers and may bind to and thus regulate the size and shape of the crystals [39]. In addition, several other substances are known to affect the growth and maturation of HAP in bone. For example, dietary cations (Mg^{2+}, Sr^{2+}), the toxic pollutant cadmium (Cd^{2+}), as well as carbonate (CO^{3-}) and citrate in the body fluids may incorporate or substitute other ion groups leading to smaller, less perfect and thus more soluble crystals. The opposite effect is caused by incorporation of fluoride (F^{1-}) which increases the crystal size thus decreases the solubility. Bisphosphonates, which are extensively used in the treatment of osteoporosis, incorporates in bone during the HAP crystal formation due to their high affinity for calcium. When bone resorption occurs, the bisphosphonates are released inhibiting osteoclast function and stimulating apoptosis and thereby further bone resorption. In addition, tetracycline binds with high affinity to the surface of recently formed HAP crystals and may be used to determine bone turnover [40].

1.3 Bone remodeling

Bone models and remodels continuous throughout life in a tightly coupled process of bone resorption and formation carried out by the osteoclasts and the osteoblasts. Bone *modeling* occurs during growth when the skeleton shapes and changes to achieve its structure and function. *Remodeling* is the continuous replacement of old bone (or cartilage) with new bone. The purpose of bone remodeling is not well known, however, removing dead osteocytes and microcracks as well as to maintain oxygen and nutrient supply to keep a healthy skeleton are likely reasons. Bone remodeling may also occur in response to hormonal regulation e.g. to release calcium and phosphate to the blood. Bone is remodeled in small units called *the bone multicellular unit* (BMU) which consists of a bone resorbing osteoclasts in front followed by bone forming osteoblasts (Fig. 6). When remodeling occurs in cortical bone the BMU (also called an osteon) excavates and replaces a canal (Haversian canal) axial to the applied

mechanical force. In cancellous bone, remodeling occurs across the trabeculaes excavating and replacing small packets of bone. At any moment more than a million BMUs are operating in the skeleton of healthy adults renewing the whole skeleton every 10 year.

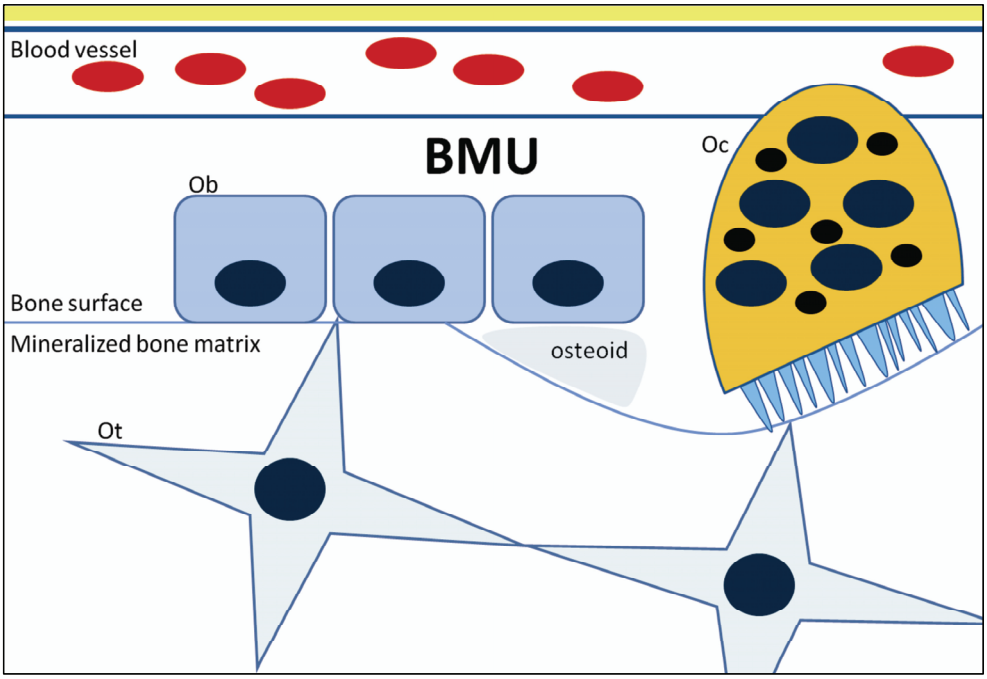


Fig. 6 Bone multicellular unit (BMU) with a leading bone resorbing osteoclast (Oc) in front followed by a team of bone forming osteoblasts (Ob). A central capillary and a nerve (in the top of the figure) are also present in the BMU.

The bone remodeling cycle consists of 3 phases: *The resorption phase* where the osteoclasts remove bone; *the reversal phase* with the formation of the "cement line", a glycoprotein-rich material laid down by what seems to be premature osteoblastic cells that gradually differentiates into bone forming osteoblasts [41]; and *the formation phase* where the mature osteoblasts form new bone. These phases occur with different speeds: resorption continues for about 14 days, reversal for 4 to 5 weeks while bone formation may take several months before a new structural unit is fully formed (reviewed in [18]). This means that if bone resorption is accelerated, a temporary imbalance occurs between resorption and formation. During the last decade it has been clarified that the osteocyte is a main orchestrator of bone remodeling by regulating both the resorption and the formation. Recent studies have demonstrated that the osteocyte is the main source of RANKL [19, 20] in adult bone. In

addition, the hypertrophic chondrocyte also express RANKL [8, 20]. This may indicate that the bone resorption process is more targeted than stochastic as the cells in or near areas in need for bone resorption are the ones initiating it. In adult bone the apoptotic osteocytes or their surviving neighbors have been suggested to determine the targeted sites for the bone resorption (reviewed in [18]). However, the remodeling sequence is regulated by several local as well as systemic factors (Table 1) and many are mediated through the RANK-RANKL-OPG axis (Fig. 4).

1.3.1 Regulation by local factors

It is well known that increases or decreases in the rate of bone resorption are tightly followed by the same response in the rate of bone formation. Over the years much attention has been paid to the "coupling" between resorption and formation, trying to identify a main "coupling-factor". The osteoclastic bone resorption releases a lot of growth factors from the mineralized matrix which stimulate osteoblast differentiation, proliferation and activation. TGF-β, IGF-I/II and the BMPs are among these. Osteoclasts may also regulate osteoblasts directly through e.g. binding of the transmembrane protein ephrin-B2 (EFNB2) to its receptor EPHB4 on osteoblasts leading to decreased osteoclast activity and increased osteoblast differentiation [42]. In addition, osteoclasts may secrete osteoblast stimulatory factors such as WNT10b and BMP6 (reviewed in [43]). Moreover, the osteocyte expresses sclerostin, the product of the SOST gene. Sclerostin inhibits bone formation via the canonical WNT/β-catenin pathway by binding to LRP5/6 which further inhibits the binding of WNTs with subsequent degradation of β-catenin in cytosol and shutdown of the differentiation from mesenchymal precursors into bone forming osteoblasts. By suppressing sclerostin (by e.g. mechanical loading or PTH) the canonical WNT/β-catenin pathway is activated and β-catenin accumulated in the nucleus stimulates osteoblast differentiation. In mature osteoblasts and osteocytes β-catenin upregulates OPG, inhibits RANKL and thereby decreases osteoclastogenesis and bone resorption. Targeted deletions in the sclerostin gene in mice (Sost) cause high bone mass and increased bone strength as does the rare disorder sclerostosis among humans (reviewed in [14, 17]). Sclerostin antibodies have shown promising results as bone anabolic therapeutics in osteoporosis as well as to improve fracture healing [44].

1.3.2 Regulation by systemic factors

Parathyroid hormone (PTH), 1,25$(OH)_2$ vitamin D_3 (1,25D), calcitonin, and estrogen are pivotal in bone remodeling, however, also other hormones including growth hormone (GH), thyroid hormone and glucocorticoids contribute to the regulation. A predominant function of PTH, 1,25D and calcitonin is to maintain adequate calcium and phosphate levels in the circulation using bone as a mineral reservoir.

PTH is secreted from the parathyroid glands and targets the kidneys as well as bone. Low levels of calcium and high levels of phosphate in the circulation stimulate PTH secretion which leads to enhanced renal tubular reabsorption of calcium and increased excretion of phosphate and bicarbonate. In addition, vitamin D is hydroxylated in the kidneys under influence of PTH to achieve its active form 1,25D. In bone PTH stimulates bone resorption via its receptors on osteoblasts and bone marrow stromal cells increasing CSF1 and RANKL and decreasing OPG (reviewed in [14]), however, data have also been reported on possible PTH receptors in osteoclasts [45]. Dependent upon mode of administration exogenous PTH has dual effects on bone remodeling; continuous PTH administration leads to increased bone resorption, while intermittent administration leads to increased bone formation. The latter may in part be due to the reduced levels of sclerostin by PTH (reviewed in [17]).

Vitamin D belongs to a group of sterols where vitamin D_2 and D_3 are the most important. It is crucial for calcium and phosphate absorption from the intestine and adequate levels of these ions have to be present to permit proper bone mineralization. Vitamin D_2 is plant-derived while vitamin D_3 is synthesized in the skin from cholesterol in response to UVB radiation, however both can be introduced through the diet. To achieve its active form, 1,25D, vitamin D needs to be hydroxylated in the liver (to calcidiol, 25D) and subsequently in the kidneys (to calcitriol, 1,25D) under stimulation of PTH for optimal function in target tissues. 1,25D acts in a negative feedback loop on PTH synthesis in the parathyroid glands as well as induces FGF23 synthesis in the osteocytes which in turn downregulates the synthesis of 1,25D in the kidney. These loops maintain a steady level of calcium and phosphate in the circulation preventing hypo- as well as hyperstates [46]. 1,25D mediates its effects via the vitamin D receptor-retinoid X receptor (VDR-RXR) complex acting directly on vitamin D responsive elements (VDRE) in the DNA of 1,25D regulated genes. In bone cells the VDR is present in osteoblasts, osteocytes and growth plate chondrocytes where the activation stimulates the expression of e.g. OCN, OPN, LRP5, and FGF23 [46-48]. At high non-physiological concentrations and under conditions with low levels of circulating calcium and

phosphate, 1,25D stimulates RANKL mediated bone resorption to mobilize ions to the circulation and represses the expression of OPG and RUNX2 [46, 47].

Calcitonin is a peptide hormone produced by the interstitial C-cells in the thyroid gland and the synthesis is stimulated by increased levels of serum calcium. Although influencing calcium homeostasis, calcitonin is less important than PTH and 1,25D and has the opposite effect by lowering the serum calcium. It inhibits osteoclastic bone resorption, however only transiently because the osteoclasts escape from the inhibitory effect after prolonged exposure probably due to down regulation of receptor mRNA [49].

Estrogen is the most important bone-sparing hormone in the skeleton for both sexes (reviewed in [43, 50]). The crucial effects of estrogen on bone tissue are illustrated by the phase of accelerated net bone loss following menopause; ovarian failure and declining estrogen levels increases bone resorption which outstrips the fine-tuned coupling to bone formation. The effects of estrogen is mainly mediated through intranuclear receptors (ERs), which are expressed in osteoblasts [51], osteocytes [52], osteoclasts [51-53] as well as in T-cells [54]. Two types of ERs, ERα and ERβ [55] are present in bone. It is considered that the bone-conserving effects of estrogen are modulated via osteocytes, which seems to be the main controller of bone remodeling. Estrogen promotes survival of osteocytes by inducing a cascade leading to inactivation of the pro-apoptotic protein BCL2-assosiated death promoter (BAD) (reviewed in [43]), while estrogen withdrawal increases osteocyte apoptosis and thereby trigger bone remodeling [56]. Estrogen also acts directly upon osteoclastic bone resorption by inhibiting activation of genes encoding important factors for osteoclasts development, such as IL6, TNF-α and CSF1 as well as suppressing TNF-α gene expression leading to decreased osteoclast number and activity [57]. Estrogen blocks the effects of RANKL and CSF1 in osteoclasts, decreases the production of RANKL by osteoblasts and T and B cells and upregulates the synthesis of OPG (reviewed in [43]) in addition to TGF-β by osteoblasts inducing osteoclast apoptosis. A recent study [58] has shown that estrogen upregulates MMP3 cleavage of FASLG in the osteoblasts membrane. This leads to increased levels of soluble FASLG inducing apoptosis via FAS in osteoclast precursors as well as in mature osteoclasts and thereby inhibiting bone resorption. Estrogen also favors net bone formation by binding to its ER receptor in osteoblasts reducing oxidative stress and apoptosis. In sum these results offer an explanation to why estrogen withdrawal is so devastating for bone mass; the increased number of bone remodeling sites due to enhanced osteocyte apoptosis and bone resorbing osteoclasts in addition to increased osteoblast apoptosis with

reduced bone formation, explain why estrogen deficiency leads to a gap between resorption and formation with a resulting net bone loss.

Table 1 Overview of some local and systemic factors acting upon bone remodeling

Factor	Bone resorption	Bone formation
BMPs	-	↑ differentiation of Ob via RUNX2
TGF-β	↓ → ↑OPG and ↓RANKL	↑ differentiation of Ob
Sclerostin	↑ → ↓OPG and ↑RANKL	↓ differentiation of Ob via ↓β-catenin
NO	-	↑ bone formation
PGE2	-	↑ bone formation
Insulin	↑ → ↓OPG and ↑RANKL	-
Leptin	↓ → ↑CART → ↓RANKL ↑ → ↑SNS →↑RANKL	↓ Ob proliferation via ↑SNS
1,25D	↑ → ↓OPG and ↑RANKL	↑ bone formation and mineralization via LRP5, OPN, OCN ↓ Ob differentiation via RUNX2
PTH	↑ → ↓OPG and ↑RANKL	(↑) intermittent → ↓ sclerostin levels
Calcitonin	↓ inhibits Oc differentiation	-
Glucocorticoids	↑ Oc survival	↓ by suppression of Ob differentiation and stimulation of Ob and Ot apoptosis
Estrogen	↓ Increases Fas induced Oc apoptosis directly and via Ob ↓ Blocks RANKL/CSF1 ↓→ ↑OPG and ↓RANKL by Ob, T and B cells	↑ bone sparing effect due to pro-survival signaling in Ot ↑ by inhibiting oxidative stress and Ob apoptosis ↑ by inhibiting sclerostin?

1.4 Bone as an endocrine organ

Recent data suggest that bone may function as an endocrine organ. Osteoblasts, in addition to its well known synthesis of collagen type I and bone matrix proteins also produce the hormone *osteocalcin* (OCN) which is carboxylated by vitamin K and stored in mineralized bone matrix. OCN is suggested to play a role in an endocrine axis where bone participates in the regulation of energy metabolism. When bone resorption occurs, OCN is undercarboxylated and activated by the acidic environment in the osteoclast resorption pit and secreted into the circulation where it stimulate the β-cells to increase insulin synthesis in the pancreas, the testosterone synthesis in male gonads, as wells as increases insulin sensitivity in muscles, adipose tissue and liver. A positive feedback loop occurs when insulin inhibits OPG in osteoblasts, up-regulates RANKL secretion and thereby bone resorption allowing more OCN to be activated and secreted into the circulation (reviewed in [14, 59]). On the other

hand, *leptin*, a hormone produced by adipose tissue, also controls the bioactivity of OCN via actions involving the sympathetic nervous system (SNS) favoring decarboxylation of OCN and thereby decreases OCN activity. In addition, leptin acts on bone metabolism via two different pathways with opposite effects that in sum prevent bone mass accumulation; it inhibits osteoblast proliferation and stimulates RANKL and bone resorption via SNS as well as decreases RANKL expression via cocaine- and amphetamine-regulated transcript (CART) (reviewed in [59]).

Another endocrine factor produced in bone is *fibroblast growth factor 23* (FGF23). FGF23 is synthesized in the osteocyte under stimulation of VDR-RXR by 1,25D and targets the kidneys where it binds to FGFR-α-Klotho complexes in kidney tubules and stimulates the elimination of phosphate as well as feedback represses the synthesis of 1,25D in the kidney. FGF23 synthesis is also enhanced by high circulating phosphate levels and decreased by PHEX and DMP1, two other osteocyte derived factors, allowing reabsorption of phosphate in the kidneys to maintain sufficient phosphate levels and subsequent mineralization of bone. High circulating FGF23 levels are associated with hypophosphatemic rickets and osteomalacia. Low levels are associated with hyperphosphatemia, increased 1,25D and ectopic calcification (reviewed in [17, 60, 61]). Interestingly, lower expression of FGF23 was observed in the fracture callus of our rats with experimental osteoporosis compared to normal controls [62].

1.5 Bone biomechanics and fracture repair

Knowledge of bone construction is essential to understand bone biomechanics. A compact cylinder is extremely strong but heavy, thus the intelligent construction of the skeleton can be illustrated by the long bones; they are thick-walled pipes possessing mechanical strength but still relatively light due to their tube form. Bone area reflects bone mineral density (BMD), but the *second moment of area* (m^4) is a property of shape that is used to predict resistance of materials to *bending* and *deflection*. In brief, assumed the same bone area, a tubular structure has greater resistance to bending than a massive cylinder due to efficient distribution of material. Increasing the diameter and decreasing the cortical wall thickness of a tube increases the second moment of area, thus increasing the resistance to bending. This can be illustrated by appositional bone growth; bone added periosteally increases the bending strength of the bone more than the increase in bone area, even if the absolute bone volume and BMD are unchanged (reviewed in [63]).

1.5.1 Biomechanical properties of bone

Materials can be described as weak or strong, ductile or brittle, stiff or compliant dependent of the outcome of a stress-strain test (e.g., the load-deflection curve obtained by loading a bone specimen until failure, Fig. 7). *Stress* is the load per unit area. *Strain* (deflection) is the percentage change in length calculated as the size of deformation divided on the original length of the specimen. The slope of the linear region of the stress-strain curve is called the *Young's modulus* of the material, representing *stiffness*; the greater the slope, the stiffer the material. The height of the curve represents the *ultimate moment* (stress), which is a measure of *strength*. The *yield point* is the point where the curve begins to bend. Prior to the yield point the bone bends elastically and will return to its original shape when the applied stress is removed. Once the yield point is passed some fraction of deformity will be permanent. The area under the curve (the integral of the function $f(x)$) is the amount of energy the tissue can resist before failure (*energy absorption*) and is also called *toughness*. Thus, a bone that is strong and stiff may require less energy to fracture (less area under the load-deflection curve) than a weak, compliant bone.

The components of bone have different impact on the mechanical properties; the intermolecular cross-links in collagen are suggested to enhance bone toughness [64, 65], while the fiber orientation is considered critically important to bone strength. The latter is supported by the fact that collagen fibers tend to be orientated axial to the applied external force [66, 67]. This can be illustrated by the congenital pathologic condition osteogenesis imperfecta, "the brittle bone disease", caused by different mutations in the genes encoding collagen type I making the affected individuals prone to low trauma-fractures. Bone mineral provides strength and stiffness, and the mineral to collagen-ratio affects both parameters. As the fraction of the mineral volume increases, Young's modulus is improved (increased stiffness), but ultimate strain (deflection) decreases and the bone becomes more brittle. The adult variant of vitamin D deficiency, osteomalacia, with failure of normal bone mineralization makes the bones more prone to deformation during loading "soft bones", thus fractures do not easily occur due to the decrease in mineral volume fraction.

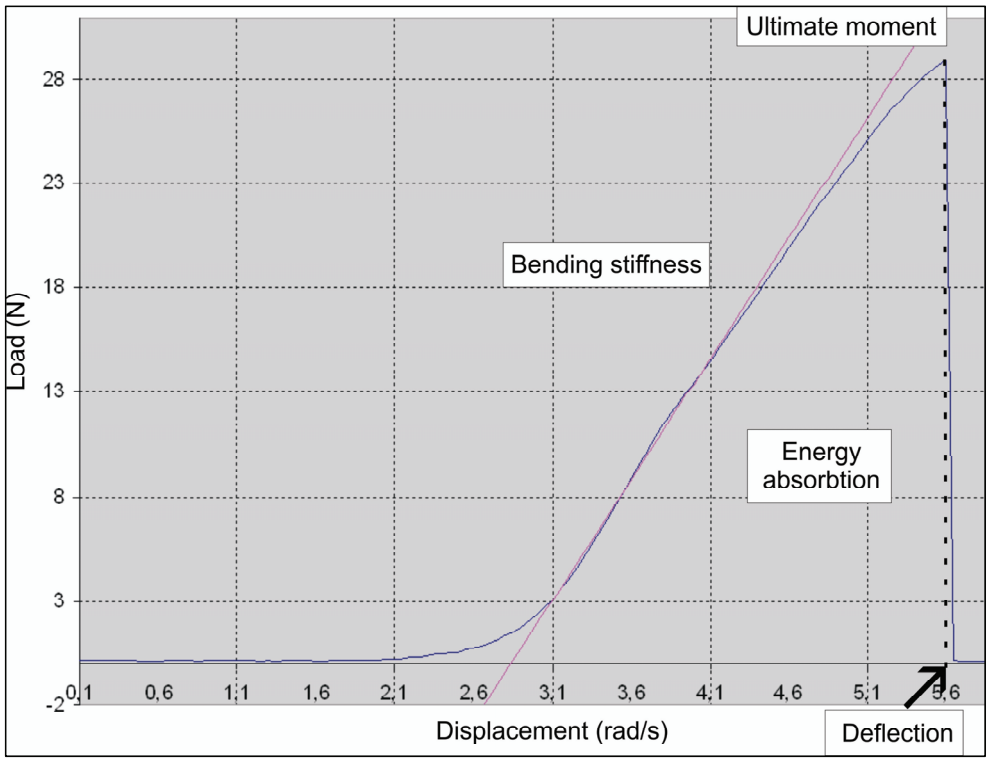


Fig. 7 Three-point cantilever bending test Load-displacement curve from mechanical testing of a rat femur demonstrating the *ultimate moment/bone strength* (top of the blue curve), *bending stiffness* (the slope of the linear region, red line), *deflection* (the ultimate deformation of the bone before fracture) and *energy absorption/bone toughness* (the integrated $f(x)$).

1.5.2 Fracture repair

A bone fracture can be defined as a discontinuity in the bone tissue as a result of mechanical injury or insufficient strength of the bone tissue itself [68]. When fracture occurs, the body responds by general wound healing processes as well as tissue specific bone repair with almost complete restoration of the original anatomy. Fracture healing includes both intramembranous and endochondral bone formation. Direct intramembranous bone formation (primary ossification) is rare and permits direct reestablishment of cortical bone by the cortical components themselves and is most likely to occur if anatomic reduction and stabile fixation has been carried out. However, there is usually some flexibility in the stabilized fracture which promotes healing via the formation of a cartilagenous template and several steps from the endochondral bone formation are recapitulated.

The fracture healing process is often divided into 4 phases: *The inflammatory phase* is the initial body response to tissue damage and is much the same as in general wound repair. A fracture hematoma is formed due to vascular damage. Platelets are activated and inflammatory cells infiltrate the hematoma which subsequently reorganizes into granulation tissue with newly formed vessels and fibroblasts laying down collagen. The hematoma with platelets and inflammatory cells is an important source of osteogenic signaling molecules resulting in recruitment of osteoprogenitor cells from the periosteum, bone marrow and the surrounding soft tissue. Multiple growth factors and cytokins coordinate the process e.g. macrophage derived growth factor (MDGF), platelet derived growth factor (PDGF) and fibroblast growth factors (FGFs) as well as members of the TGF-β superfamily, interleukins and BMPs are involved (reviewed in [69]). The central role of the inflammatory phase can be illustrated by the effect of anti-inflammatory agents, such as non-steroidal anti-inflammatory drugs (NSAIDs), resulting in increased fibrinogenesis, decreased bone formation and delayed fracture healing (reviewed in [70]). *The formation of soft callus* is the start of the reparative phases which is dominated by the formation of a cartilaginous callus promoting early stabilization to the fracture site and serving as a template for the later bony callus formation. This is much a repetition of the endochondral bone formation process with mesenchymal precursor cells differentiating into chondrocytes and hypertrophic chondrocytes with cartilage maturation and mineralization [71] (see 1.1.1 for details). *The formation of hard callus* describes the osteogenesis which also occurs during direct, primary ossification if the fracture stabilization is sufficiently rigid. As soon as the cartilage is mineralized the in-growth of vessels allows the osteoprogenitors to proliferate and differentiate into bone-forming osteoblasts synthesizing new, irregular bone (woven bone). The hypertrophic chondrocytes stimulate differentiation of chondroclasts/osteoclasts by increasing levels of CSF1 and RANKL promoting resorption of the mineralized cartilage, while members of the BMP family promote recruitment of the osteoprogenitors and VEGF stimulates neoangiogenesis (reviewed in [69, 72]). *The remodeling phase* begins when the cartilage is resorbed and the callus almost exclusively consists of woven bone (after approximately 3-6 weeks). The final remodeling of the callus from woven bone into the original cortical or trabecular configuration with the re-establishment of the marrow cavity, may take months or even years and is driven by a coupled process of osteoclastic bone resorption and osteoblastic bone formation similar to regular bone remodeling.

1.6 Osteoporosis

Osteoporosis (greek; ostoun meaning bone and poros meaning pore) is an age-related bone disorder affecting both men and women where imbalance between bone resorption and formation leads to a net bone loss with resulting bone fragility and increased fracture risk. BMD is reduced as a measure of bone loss and the bone microarchitecture is deteriorated. Osteoporosis is the most frequent bone disorder in the world and the most common metabolic disorder among the elderly (reviewed in [73]). The condition is a major health problem not only for the affected individuals but also for the society. In Norway the costs of hip fractures alone has been estimated to 2.0 billion NOK a year [74]. Norway and Sweden as well as northern parts of the United States have the highest prevalence of osteoporosis worldwide [75], and although the incidence of hip fractures in Norway has decreased over the last decade it is still the highest in the world [76]. As the populations in the western world are getting older fragility fractures are expected to increase although the age-related incidence is unchanged or decreasing. The World Health Organization (WHO) has defined osteoporosis in both men and women as BMD values 2.5 standard deviations or more below the BMD for the young, healthy female Caucasian measured by dual-energy X-ray absorbtiometry (DEXA/DXA) [77]. Established osteoporosis includes, in addition to low BMD, the presence of a fragility fracture and the hip, distal forearm, spine and proximal humerus are especially prone to such fractures. However, BMD alone is not sufficient to predict the risk of a future fragility fracture, and the WHO therefore recommend the use of FRAX (www.shef.ac.uk/FRAX/tool.jsp) which is the WHOs fracture risk assessment tool. This tool incorporates several known risk factors for osteoporosis and fractures including BMD, and predicts the patient's 10 year probability of fracture. The set up also contains recommendations for anti-osteoporotic therapy.

New perspectives on osteoporosis as a part of the frailty syndrome classify osteoporosis as a degenerative disorder in line with atherosclerosis, myocardial hypertrophy, insulin resistance and Alzheimer's disease. This has lead to a focus shift from the "estrogenocentric" view highlighting postmenopausal estrogen deficiency towards increases in cellular oxidative stress being the main pathological mechanism causing osteoporosis (reviewed in [73]). However, it is well documented that an accelerated rate of bone loss is taking place right after menopause [78] due to the withdrawal of estrogen and the hormone's bone-sparing "anti-oxidative" effects.

Bone loss begins in the thirties for both men and women. After reaching the peak bone mass in the early thirties, the continuous remodeling of the skeleton slowly looses bone as the defense against oxidative stress in the organism is reduced. Consequently, individuals with low peak bone mass are more prone to osteoporosis and fragility fractures. Low peak bone mass is influenced by several genetic and non-genetic factors; among the genetic determinants are female sex, body mass index (BMI) and age at menarche [79] in addition to polymorphisms in genes regulating bone mass e.g., VDR [80], COLIA1 [81], the genes influencing the WNT/β-catenin pathway such as the LRP5, DKK and SOST [44, 82, 83] as well as some more un-known genes associated with BMD [83]. The non-genetic factors contributing to low peak bone mass are e.g. low daily calcium intake during childhood, low body weight, sedentary lifestyle and delayed puberty [84, 85] as well as some therapeutics e.g. glucocorticoids and anti-convulsives.

1.7 Vitamin D deficiency

Vitamin D deficiency may be due to inadequate intake or absorption from the intestine, defects in the synthesis, or vitamin D resistance in the target tissues (reviewed in [86]). Vitamin D inadequacy is common among the elderly [87, 88] leading to decreased intestinal calcium absorption and increased levels of PTH (secondary hyperparathyroidism). This results in enhanced bone turnover as well as fatigue and muscle wakening increasing the risk for falls. Severe vitamin D-deficiency during childhood or in the adults manifests in bone as either *rickets* or *osteomalacia,* respectively, due to the insufficient mineralization of osteoid and/or the periosteal/endosteal appositional growth. In children, rickets includes failure of and/or delayed mineralization of growth cartilage at the epiphyseal growth plate which becomes thick, wide and irregular. Clinically rickets/osteomalacia manifest with skeletal deformities, bone pain and pseudofractures (reviewed in [89]).

1.8 Tartrate-resistant acid phosphatase

Tartrate-resistant acid phosphatase (TRAP) (ACP5), also known as purple acid phosphatase, or uteroferrin [90], is a well known enzyme demonstrated in many mammalian tissues [91-93], and most known as an osteoclast marker. Acid phosphatase activity in bone was described as early as in 1969 [94] and in 1977 it was confirmed that there are 2 types of acid phosphatases (ACPases) in bone tissue; tartrate-sensitive ACPase and tartrate-resistant ACPase [95]. TRAP belongs to the subfamily of purple acid phosphateses (PAP), a group of

metalloenzymes [96] which catalyzes hydrolysis of phosphate esters and anhydrides under acidic conditions. Mammalian TRAP enzymes are glycoproteins with a molecular weight of approximately 35 kDa. TRAP is synthesized as a relatively inactive pro-enzyme (monomeric TRAP/mTRAP/loop-TRAP/serum TRAP 5a) and proteolytic cleavage by members of the cathepsin family or other proteinases, increases the catalytic activity at least 10-fold [97, 98]. TRAP gene expression seems to be most abundant in bone tissue [99] where it has shown several biological abilities; e.g., dephosphorylation of OPN and IBSP [100, 101], and of Man-6-P recognition marker on lysosomal proteins [102, 103] as well as generation of reactive oxygen species (ROS) [35] for bone matrix degradation. Human serum contains two isoforms of TRAP; 5a and 5b with different pH optimum; 5.0-5.2 and 5.8-6.0 respectively. Serum TRAP 5a is identical to monomeric TRAP, while serum TRAP 5b is identical to osteoclastic, cleaved TRAP (reviewed in [104]). The serum activity of TRAP 5b is significantly elevated in patients with osteoporosis and negatively correlated with BMD [105]. Studies on mice have yielded similar results: overexpression of TRAP results in enhanced bone turnover and a mild osteoporotic phenotype [106], while global deletion of TRAP leads to disturbed endochondral ossification with widening of the growth plate as well as an enhanced number of thickened trabeculae in the metaphysis and a mild osteopetrotic phenotype [107-109].

TRAP has also been reported in osteoblasts and osteocytes [94, 110-117]; TRAP mRNA and enzyme activity have been demonstrated in the metaphysis and in the endosteal and periosteal aspects of diaphyseal rat bone [110, 116] as well as in osteocytes in diaphyseal rat bone near bone resorption sites [110, 113]. The origin and function of TRAP in these cells have been debated; one hypothesis is that osteoclastic TRAP from the resorption lacunae is endocytosed by osteoblasts. This theory is supported by cell culture studies reporting that osteoblast-like cells are able to engulf osteoclastic TRAP and inactivate the enzyme, suggesting that this could control the enzyme activity and prevent further degradation of matrix constituents [114, 115]. However, endogenous TRAP production has also been demonstrated in osteoblasts [116] and osteocytes [113] in areas close to bone resorbing surfaces suggesting that TRAP may take part in mechanisms controlling the direction of osteoclastic bone resorption [113]. There are also *in vitro* results indicating that TRAP in the osteoclast lacunae may play a role in the signaling pathway responsible for the differentiation of osteoblast-like cells into mature osteoblasts and thereby being one of the “coupling-factors” between bone resorption and bone formation [118]. In addition, increased amounts of TRAP and cathepsin K have been demonstrated in large osteocyte lacunae and canaliculi in lactating mice, suggesting that osteocytes are able to remodel their matrix via osteoclast-like

mechanisms under specific conditions [21]. Despite excessive studies on TRAP in bone, the exact function(s) still remains elusive.

2 AIMS OF THE STUDIES

In the current project the initial goal was to establish an animal model for studies of fracture healing in osteoporosis as well as to use animal models with disturbed bone turnover to provide new insight into bone metabolism at the molecular level.

Paper I

In the first paper we aimed to establish the experimental osteoporosis model by the combination of ovariectomy and vitamin D deficiency in rats, and use this model to test the hypothesis that experimental osteoporosis delays bone repair manifested as impaired mechanical properties and bone mineral density of the fracture callus.

Paper II & III

In paper II we wanted to test the hypothesis that tartrate-resistant acid phosphatase (TRAP) in osteoblasts and/or osteocytes in rat bone tissue mainly has an exogenous osteoclastic origin *in vivo* by demonstrating enhanced levels of TRAP in such cells in animal models with increased osteoclast activity; i.e. in experimental osteoporosis and in experimental rickets. The second hypothesis was that endocytosed TRAP in osteoblasts and/or osteocytes will locate to late endosomes or lysosomal structures for subsequent inactivation and degradation or further signaling (cf. Fig. 8).

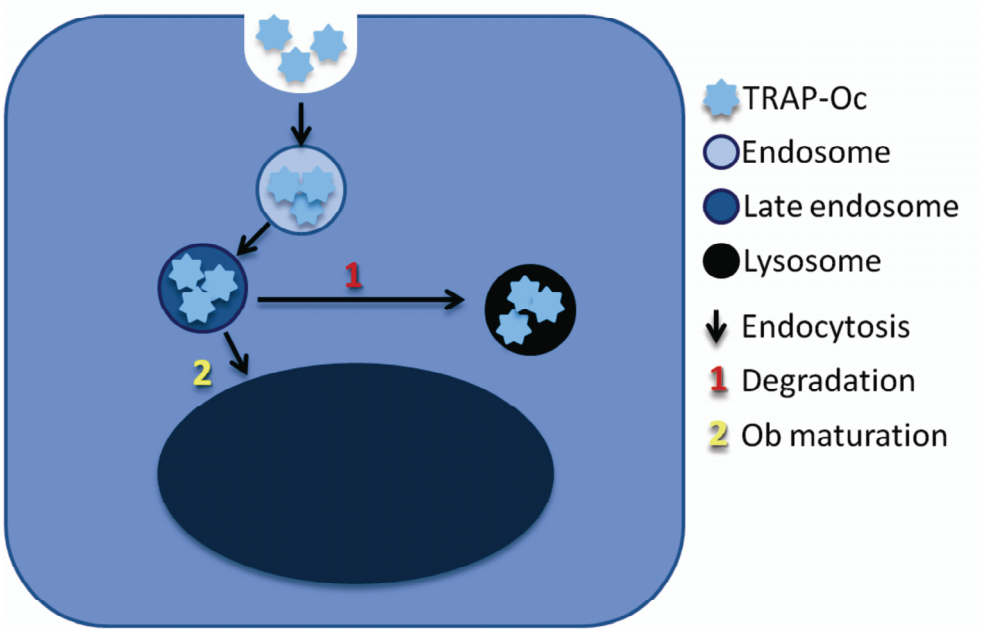


Fig. 8 The endocytosis theory for TRAP in osteoblasts A schematic drawing of the suggested routes for osteoclastic TRAP (TRAP-Oc) which is endocytosed by osteoblasts for either lysosomal degradation or for serving as a coupling factor promoting Ob maturation.

3 MATERIALS AND METHODS

An overview of the animal models and the methods applied in the included papers are given below. The reader is referred to the respective papers for further details.

3.1 Animal models and study designs

Local guidelines as well as the American Guide for the Care and Use of Laboratory Animals [119, 120] were followed and the protocols approved by the Norwegian National Animal Research Authority for the animal experiments included in this thesis. The numbers of animals were kept to a minimum in accordance to the statistical power analyses. The surgical procedures (ovariectomy and induction of a standardized fracture) were carried out under deep anesthesia and all animals received subcutaneous analgesics after the procedures and the first postoperative day. Blood samples were collected from the animals in paper I and II prior to killing. During the *in vivo* experiments all the animals were housed under standard laboratory conditions (except for the rats with experimental rickets which were kept in a room with UV-free light) in a well equipped animal laboratory (Department of Comparative Medicine, Oslo University Hospital, Rikshospitalet). The animals were taken daily care of by professionals as well as the researchers during the experiments. The animals gained weight during the course of the *in vivo* experiments and killing of all animals was performed either under deep anesthesia and *in vivo* perfusion through the hearth, by a phenobarbital overdose or by a guillotine.

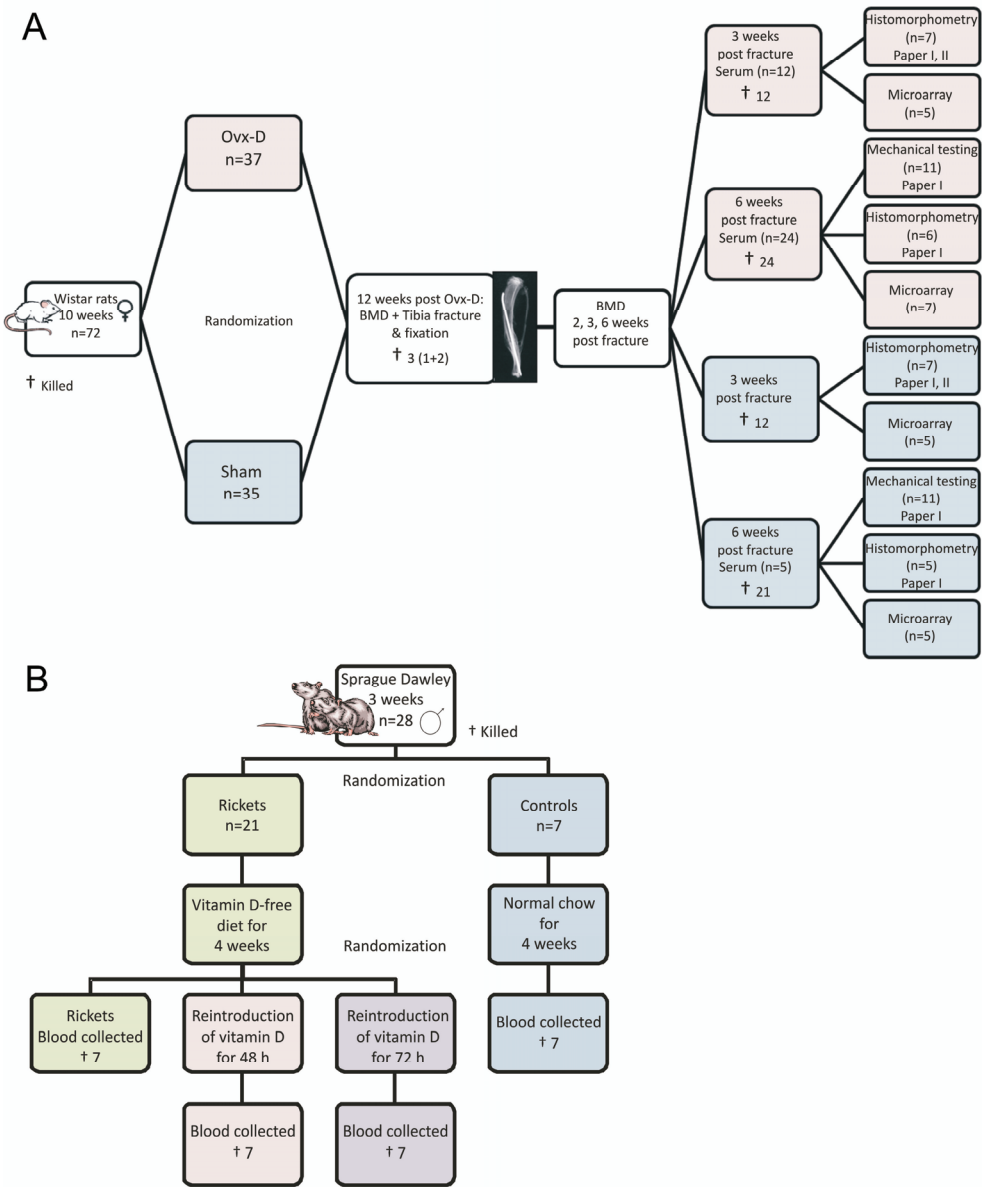


Fig. 9 Schematic diagram of the study designs of the animal models
A Experimental osteoporosis **B** Experimental rickets

3.2 Methods

Table 2 Overview of the applied methods

Paper	Animal models	Methods
I	Experimental osteoporosis: ovariectomy and vitamin D deficiency Bone repair: tibia midshaft fracture	Immunoassays for serum analyses (25OHD, estradiol) DXA *in vivo* for BMD measurements Cantilever bending test Bone histomorphometry
II	Experimental osteoporosis: ovariectomy and vitamin D deficiency Experimental rickets: low phosphate and vitamin D deficiency rickets and healing from the condition for 48h and 72h	Histology: experimental rickets Immunoassays for serum analyses (TRAP5b, CTX-I) *In situ* hybridization (TRAP) Immunolabeling and fluorescence microscopy (mTRAP, ELF97) Immunoelectron microscopy (TRAP) Semi-quantitative evaluation
III	3 days old, untreated rats	Immunofluorescence confocal microscopy and co-localization analyses (TRAP, RANKL, OPG) Immunoelectron microscopy of thawed cryosections (TRAP, RANKL, LAMP1)

4 SUMMARY OF RESULTS

4.1 Paper I

Melhus G*, Solberg LB*, Dimmen S, Madsen JE, Nordsletten L, Reinholt FP. Experimental osteoporosis induced by ovariectomy and vitamin D deficient diet does not markedly affect fracture healing in rats
Acta Orthop 2007 Jun;78(3):393-403 *The authors contributed equally to the study

Animals subjected to ovariectomy and vitamin D-depletion (Ovx-D) developed vitamin D-deficiency and presented serum levels of estradiol in the range of a human postmenopausal state. This confirmed a successful accomplishment of the intervention. The Ovx-D animals also developed osteopenia compared to the controls (sham) with significantly reduced BV/TV in the femur head ($p<0.001$), and significantly decreased BMD in the trabecular areas (spine and femoral neck, $p<0.001$). A standardized midshaft fracture was induced in the right tibia in all animals 12 weeks post ovariectomy and vitamin D-depletion. DXA-analyses at 2, 3 and 6 weeks post fracture demonstrated significant lower BMD in the right tibia midshaft at 6 weeks post fracture and in the right femur neck of both groups at all time-points compared with the left limb. No differences were detected with respect to BMD or mechanical properties of the callus between the two groups in the fractured right limb; however both groups presented a high proportion of non-unions. No impairment in bone strength was detected in the Ovx-D group compared to sham, except for significant decreased bending stiffness in the femur neck in Ovx-D. Significant weight gain were seen in the Ovx-D vs. sham despite pair-feeding ($p<0.001$), however, normalization for body weight in the mechanical test results did not alter the statistical outcome. In conclusion: experimental osteoporosis does not affect the BMD or impair the mechanical properties of the fracture callus after 6 weeks of healing.

4.2 Paper II

Solberg LB, Brorson SH, Stordalen GA, Bækkevold E, Andersson G, Reinholt FP. Increased tartrate-resistant acid phosphatase (TRAP) expression in osteoblasts and osteocytes in experimental osteoporosis in rats

Submitted October 2013

Tartrate-resistant acid phosphatase (TRAP) is a well known osteoclast marker; however, osteoblasts and osteocytes close to bone surfaces or intracortical remodeling sites also demonstrate TRAP gene and protein expression as well as enzyme activity. The origin and function of TRAP in these cells are not known, thus a suggested mechanism is that osteoblasts and/or osteocytes engulf osteoclastic TRAP for inactivation to prevent further bone resorption. To evaluate whether changes in osteoclast activity could alter TRAP protein expression and enzyme activity in osteoblasts and/or osteocytes *in vivo,* we analyzed two experimental rat models with disturbed bone metabolism and increased osteoclast activity: the ovariectomized and vitamin D-depleted rat (Ovx-D) and rats healing from experimental rickets for 48h and 72h (experimental rickets). Osteoblasts and osteocytes close to intracortical remodeling sites and bone surfaces demonstrated TRAP, most prominent in cancellous bone and in osteocytes in both animal models. Intracellular TRAP was located to electron dense vesicles with similar morphological features in both osteoblasts and osteocytes. Ovx-D increased osteoclast activity ($p<0.001$) and ELF97+ osteocytes ($p<0.05$) in cancellous bone, but no corresponding increase was observed in the osteocyte lacunar area. The ratio of TRAP+ vesicles to total cytoplasm area in cortical osteoblasts ($p<0.01$) was also increased. In addition, Ovx-D presented increased TRAP mRNA expression in osteocytes, however not significant, questioning the hypothesis of endocytosis being the mechanism enhancing TRAP protein expression and enzyme activity in osteoblasts and osteocytes. To further address this question, rats healing from nutritionally induced low phosphate and vitamin D-deficiency rickets (experimental rickets) were analyzed as an alternate model of osteoclast activation. Enhanced osteoclast activity was noted in healing rickets after 72h ($p<0.05$), but no differences in TRAP expression were detected in osteoblasts or osteocytes. In conclusion; increased osteoclast activity does not affect TRAP expression in osteoblast and osteocytes favoring the notion that the observed increase in TRAP protein expression and enzyme activity in osteoblasts and osteocytes is due to increased synthesis rather than being a result of increased osteoclast activity.

4.3 Paper III

Solberg LB, Stang E, Brorson SH, Andersson G, Reinholt FP. Co-localization of tartrate-resistant acid phosphatase (TRAP) and receptor activator of NFKB ligand (RANKL) in lysosomal associated membrane protein 1 (LAMP1) positive vesicles in osteoblasts and osteocytes in rats

Manuscript November 2013

Tartrate-resistant acid phosphatase (TRAP) gene and protein expression as well as enzyme activity have previously been observed in osteoclasts, osteoblasts and osteocytes in bone tissue *in vivo* in addition to some reports on TRAP gene expression in hypertrophic chondrocytes. Paper II demonstrates enhanced level of TRAP+ osteocytes and TRAP located to intracellular vesicles in osteoblasts and osteocytes in experimental osteoporosis, especially abundant in osteocytes and osteoblasts in cancellous bone and close to bone surface and intracortical remodeling sites. To further address the function of TRAP in osteoblasts and osteocytes, long bones of young growing rats were examined in order to reveal the nature of the TRAP+ vesicles. Immunofluorescence confocal microscopy displayed co-localization of TRAP with RANKL and OPG in hypertrophic chondrocytes and diaphyseal osteocytes. Pearson's correlation coefficient (PCC) $\geq$ 0.8 indicated strong co-localization of TRAP with RANKL and OPG in hypertrophic chondrocytes as well as in diaphyseal osteocytes. Transmission electron microscopy (TEM) of tibia metaphysis and diaphysis demonstrated TRAP and RANKL in LAMP1+ electron dense vesicles in osteoblasts and osteocytes and also displayed co-localization of TRAP and RANKL in vesicular structures in both cell types. These observations support the results obtained by confocal microscopy. Recently, *in vitro* data have been reported on OPG as a traffic regulator for RANKL to LAMP1+ secretory lysosomes in osteoblasts and osteocytes serving as temporary storage compartments for RANKL. In addition, hypertrophic chondrocytes express RANKL and OPG. We demonstrated co-localization of TRAP with RANKL in LAMP1+ vesicles in osteoblasts and osteocytes as well as co-localization of TRAP with RANKL and OPG in hypertrophic chondrocytes and osteocytes. In conclusion: Our observations of RANKL and LAMP1 in TRAP+ vesicles in osteoblasts and osteocytes *in vivo* indicate that TRAP is located to RANKL positive secretory lysosomes in osteoblasts and osteocytes.

5 GENERAL DISCUSSION

5.1 Methodological considerations

5.1.1 Animals

In vivo experiments as well as studies of cell cultures have extended our knowledge on bone metabolism and bone repair. New intracellular pathways and mechanisms are often discovered *in vitro*; however, there is also a need to confirm the presence and importance of such mechanisms *in vivo* before carrying out further research that eventually may end up in large scale studies of promising new therapeutics. Experimental studies on bone metabolism and bone repair in a randomized controlled fashion are difficult to perform in humans, and cell culture studies will not let us examine the *in vivo* aspects, we therefore turned to the laboratory rat as model for *in vivo* experimental studies. The animal models described in this thesis have previously been shown to be useful for studying different aspects of bone tissue properties and are published by other associated member of our group [121-126].

Rats are widely used in bone research and their anatomy and physiology are well known. In addition, they are easy to house, low at cost and numerous experiments have been carried out in bone making the comparison between different studies easy. However, the use of rats in experimental bone research do have some limitations: rodent bone structure is more primitive than in humans, lacking Haversian systems in cortical bone and intracortical remodeling in young rodents are carried out in resorption cavities [127]. This process shows similarities with the Haversian remodeling occurring after fracture healing in humans, and the use of rats in studies of bone repair has been accepted [128] as long as the limitations are taken into account when extrapolating the results to humans. The lack of Haversian systems may also be a potential drawback when inducing osteoporosis in rats, as cortical porosity in human osteoporosis is due to increased Haversian remodeling. However, the induction of osteoporosis in rats by surgical ovariectomy leads to a condition similar to the rapid bone loss observed right after menopause in human females [129]; cancellous bone mass at discrete sites exhibits an increased rate of bone remodeling and an altered balance between resorption and formation occurs. This favors resorption with trabecular thinning and cancellous bone loss, while in cortical bone the mid-diaphyseal diameter increases by periosteal growth as well as endosteal thinning. The net result is enlargement of the medullary cavity which may be observed at earliest between 90 and 120 days after ovariectomy [130]. An additional

difference between human and rat bone biology is the persistent longitudinal long bone growth with late closure of the growth plates occuring in rats: they reach their sexual maturity at the age of 2.5 months and their peak bone mass and skeletal maturity at 10 months. However, some of the epiphyseal growth plates remain open after this; in male rats the epiphyses of long bones stay open until past 30 months, while in female rats the proximal tibia epiphyses close at 15 months [129]. This makes it mandatory to use a control group to be able to differentiate the gain or loss of bone mass from the natural bone modeling, and to be aware of the growth and maturation rate for the discrete skeletal sites evaluated. Furthermore, ovariectomized rats are not prone to fragility fractures, which is the main symptom of human osteoporosis. Fragility fractures or spontaneous fractures have actually never been reproduced in any animal model. The WHOs definition of osteoporosis, i.e. BMD below 2.5 SDs of the reference group with or without fractures, might be used in animal models. Moreover, WHO has accepted the osteopenic rat without fragility fractures appropriate for osteoporosis research [129]. Also guidelines from the Food and Drug Administration in the US (FDA) support this notion by recommending the ovariectomized rat as one out of 2 animal models (the other being a large animal model) for studying the effect of drugs affecting postmenopausal osteoporosis in proximal tibia, distal femur and lumbar vertebrae [131].

5.1.2 The experimental osteoporosis model

We induced experimental osteoporosis by a combination of ovariectomy and vitamin D deficiency (Ovx-D) in young female rats. The ovariectomy model was chosen following the reasoning above and in trying to come even closer in mimicking human postmenopausal osteoporosis, vitamin D depletion was introduced to the ovariectomized rats as low levels of vitamin D have been associated with increased risk of hip fractures [87, 132]. In addition, the model has previously demonstrated reduced mechanical strength of the femur neck [124]. Young, skeletal immature rats with a control group were chosen to be sure that an eventual delay in the fracture healing process was due to osteoporosis and not to aging which is known to impair the healing properties [133-135]. Pair-feeding was introduced to the Ovx-D animals in order to prevent the weight gain known to occur in ovariectomized rats [136] (reviewed in [137]), however, despite pair-feeding the Ovx-D animals gained significantly more weight than the sham animals. This could, at least to some extent, counteract the bone loss induced by ovariectomy. On the other hand, normalizing for body weight differences in the mechanical test results did not alter the statistical outcome.

5.1.3 The experimental fracture model

The tibia mid-shaft fracture model in rats has been widely used by associated members of our group [121, 122, 125, 126] and standardization with respect to fracture site and stabilization is well established. However, the sparsely and slowly developing cortical bone loss known to occur in the ovariectomized rat [138] may question the chosen mid-diaphyseal fracture: a metaphyseal fracture location might have been a better choice as human osteoporotic fractures are most frequently located to sites with high degrees of cancellous bone (as the spine, hip, distal forearm and proximal humerus). On the other hand, the main purpose of the study was to investigate fracture healing and the biomechanical properties of the callus rather than the biomechanical strength of long bone diaphyses in experimental osteoporosis. Assuming that the systemic effects of ovariectomy and vitamin D depletion on callus formation are independent of the fracture site and following the reasoning above, we determined the tibia mid-diaphyseal fracture model suitable for the study.

It is well known that the immobilization and fixation of the fracture site influence the degree of callus formation and the mode of fracture healing i.e. intramembranous or direct fracture healing vs. endochondral or indirect fracture healing (see 1.6.2. for details). It is therefore particularly important to be able to control the standardization and biomechanical properties of the fixation in studies of fracture healing. In our study the tibia fracture was stabilized with an intramedullary pin. According to a recent review [128], the applied stabilization method is judged both easy and reproducible. However, the method lacks rotational and axial stability and has a high risk for dislocations. Thus it is no longer recommended, as new improved, specially designed rodent implants have been made available during the last years. This was not the case at the time our study was carried out and the axial and rotational movement between the fracture-ends might have influenced the results by leading to the high degree of non-unions observed (45% vs. 36% non-unions in the Ovx-D and sham group, respectively). The non-unions were excluded from the mechanical testing, however, the remaining number of animals (6 and 7) were still sufficient to allow detection of a 20% difference in means [139].

5.1.4 Rats healing from low phosphate and vitamin D-deficiency rickets

Low phosphate and vitamin D-deficiency rickets as well as healing from the condition has previously been used for the study of chondroclasts/osteoclast resorption activity [140] and activation in rats [123]. Vitamin D-deficiency and low phosphate concentrations induce

rickets with well known morphological changes; inadequate calcification of the growth plate region with impaired hypertrophic chondrocyte apoptosis and minimal cartilage resorption result in the characteristic widening of the growth plate. The adjacent metaphysis and cortical bone also fail to mineralize properly (reviewed in [141]). Inadequacy in the circulating mineral concentration as well as reduced transcription of osteopontin [46, 142, 143] as a result of the vitamin D deficiency may contribute to the defect mineralization and the subsequent failure in resorption. The theory behind this is that the chondroclasts/osteoclasts recruited to areas in need for resorption fail to develop appropriate resorption machinery [140] with impaired polarization and ruffled border development. This might be due to the inappropriate ECM-cell interaction which seems to be essential for the correct development of ruffled border (i.e. the lack of osteopontin and defect binding to αvβ3-integrins [30, 31]). However, the exact mechanism remains to be set. Nevertheless, reintroduction of vitamin D and phosphate to the diet normalize the growth plate region by stimulating cartilage and bone mineralization, as well as increasing the chondroclast/osteoclast activity and resorption [123]. We confirmed the intended increase in chondroclast/osteoclast activity in our study by morphological examination of the growth plate region and by measuring the serum levels of TRAP 5b and CTX-I, widely used to determine osteoclast number and activity, respectively [144]. To be sure that we determined the true osteoclast activity and that the results were not influenced by e.g. differences in bone size; we calculated the ratio of osteoclast activity (CTX-I) over osteoclast number (TRAP5b) in the experimental rickets model. This is in parallel to estimating osteoclast activity by calculating the ratio of osteoclasts number per bone surface (N.Oc/B.Pm) vs. total number of osteoclasts (N.Oc/T.Ar) [145] as performed for the Ovx-D rats.

5.1.5 The applied methods

Dual energy x-ray absorptiometry and bone mineral density

A keystone in non-invasive monitoring of bone mass and prediction of fracture risk *in vivo* is the assessment of BMD by dual energy x-ray absorptiometry (DXA) measurements. BMD is not a measure of true volumetric bone density but rather a measure of mass per area. Therefore, some scientists recommend the use of 3-dimensional tools such as pQCT or μCT to obtain detailed images of the bone structure and thus allow differing between e.g. cortical and trabecular bone [129, 146], however, the limited accessibility of such hardware has previously made it difficult to use pQCT or μCT for *in vivo* measurements. In the first study

BMD was used for the evaluation of bone loss and fracture repair by repeated *in vivo* measurements of the animals pre fracture and at 2, 3 and 6 weeks post fracture. By using Lunar PIXImus (Lunar, Madison, Wisconsin, USA), specially designed for the assessment of BMD in small animals, we were able to perform the measurements *in vivo* during the healing process and thereby reducing the amount of animals needed for the study and/or increased the observation time-points.

Mechanical testing

Mechanical testing *ex vivo* is used to monitor fracture healing in order to evaluate the different biomechanical properties of the callus area. Several types of mechanical tests have been described in the literature, but tension, torsion and bending tests are the most common and possessing different advantages/disadvantages [147, 148]. *Tension tests* (longitudinal deformation of an elastic body that results in its elongation) are suggested to be most useful during the initial phases of fracture healing [149, 150]. Although *torsion tests* (twisting of a body by two equal and opposite torques) have the advantages of evaluating mechanical properties of the entire healing bone and are capable of indicating the weakest section [149], *bending tests* have proven to be useful and sensitive in evaluating mechanical properties of callus in long bones of several animal models, including rats [147, 151]. Bending tests can be performed as 3-point or 4-point variants. Three-point testing is the most widely used; it is easy to perform and is considered to be a good test for examining the properties of tibia fractures [147]. The whole bone is loaded until failure during testing. The drawback is that it creates high shear stress near the midsection of the bone. In paper I, a mechanical test capable of examining different bone sites was required. The cantilever bending test with a 3-point bending for femur shaft in addition to a modified test for the femur neck, has previously been reported successful [124] and thus chosen in order to examine the healing tibia fracture as well as the intact femur shaft and neck. Unfortunately, the measurements presented large SDs, most pronounced in the testing of the callus area, probably due to the lack of stability and reproducibility in the fracture fixation. On the other hand, the intact bones also presented large SDs and this may, at least in part, be related to the design of the bending test, the test-machine itself or the operator's skills. Another test-machine or mechanical test may have given more consistent results; a 3-point bending test has the disadvantage of the manual positioning of the fulcrum to the callus area which thereby determines the fracture point. This may lead to inadequate and less reproducible results and thus a 4-point bending test or a torsion test may have been a better choice. Another aspect is the operator's testing skills as

well as the orientation of the specimens in the test machine. However, all bending tests were performed by the same operators trained in the procedure to minimize this type of bias.

In situ hybridization

In paper II the amount of osteocytes synthesizing TRAP were analyzed by *in situ* hybridization. This is a reliable method for the morphological localization of mRNA transcripts in tissue sections *in vivo* by a complementary, labeled nucleic acid probe [152]. An advantage of *in situ* hybridization is the spatial resolution of transcript distribution that is obtained at the cellular level, and which is not achieved by northern blotting or different PCR methods. A disadvantage is that the preparation for the method is tedious and lengthy. The use of the sense probe as negative control for the staining procedure has been a common practice, however, it has been reported that anti-sense transcripts occur naturally in the tissues which may disturb the interpretation of the results. It is therefore preferable to use other genes known to have a different expression patterns as controls [152]. However, this was not judged to be a problem in our procedure.

Immunohistochemistry

The principle of all immuno analyses is the detection of antigen epitopes by antibodies which recognize and binds to one or more specific area of the epitope. The antibody is then visualized by e.g. chromogens, fluorochromes or conjugated gold (for TEM) making it accessible for analyses (Fig. 10). The main purpose of immunohistochemistry is to locate defined antigens by combining immunolabeling and morphology at the light, confocal or electron microscopic level. However, it is important to notice that false-positive as well as false-negative labeling may occur. Lack of staining does not exclude the presence of the antigen. To control false-positive staining, non-specific labeling with immunoglobulins (IgG) is commonly used together with the secondary antibody or just the solely secondary antibody. However, the best available method is labeling of sections known to be devoid of the target antigen [153], although, this is not always possible. In our studies we used non-specific IgG as well as the solely secondary antibody to control false-positive labeling. Although, it is difficult to control false-negative labeling; tissue sections known to be positive for the target antigen, e.g. TRAP in osteoclasts, were used to ensure proper antibody labeling.

An additional problem to be aware of when labeling for two or more different antibodies at the same time, is cross-reactivity between the antibodies, especially if the antibodies are raised in the same animal species. If antibodies from different animal species

are used for the double labeling, these are usually easily distinguished by species-specific immunoglobulins [154]. However, it is always important to control for cross-reactivity by single labeling for each of the antibody to ensure the labeling pattern before dual labeling is performed. We performed single labeling for every antibody subjected to dual labeling in paper III.

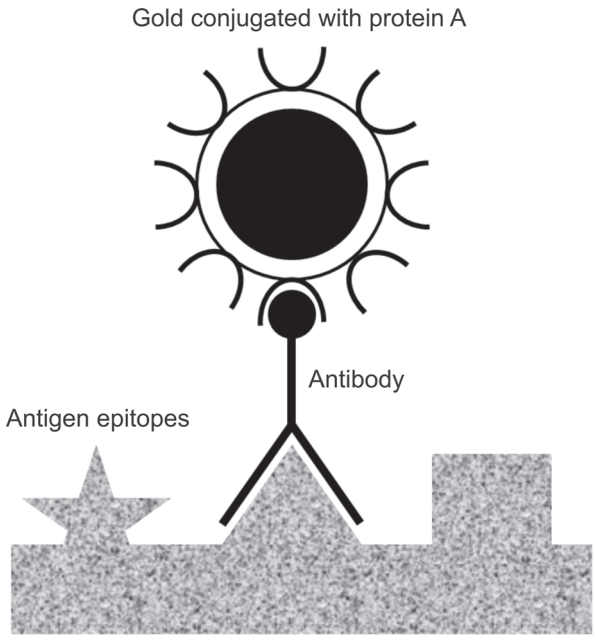


Fig. 10 Schematic drawing of the antigen-antibody interaction visualized by protein A conjugated gold particle.

Tissue preparation with chemical fixatives

Several steps during tissue fixation and preparation may influence the degree of antigen labeling; e.g. *in vivo* or *in vitro* pre-fixation, post-fixation, decalcification of bone tissue, embedding, sectioning and eventually the labeling procedure. Chemical treatment may mask epitopes and lower the antigenicity in addition to change the ratio of available epitopes between the different tissue compartments. Tissue fixed without or with limited use of chemical fixatives are closer to "nature" and have a higher degree of available epitopes, although, they are often more unstable to work with. In a previous published paper [155] we looked into the effect of a chemical fixative, paraformaldehyde (PF), on bone tissue and studied the effect of antigen retrieval by heating in order to further improve the immunogold signal. In addition, we compared the PF fixed tissue with high-pressure freezing and freeze-substitution (HPF-FS), a procedure with limited subjection to aldehyde fixation and

considered to be the “gold standard” for optimal preservation of tissue for ultrastructural examination [156-158]. PF fixed tissue, which is known to induce cross-links between the fixative and the tissue proteins [159] masking the epitopes, showed significantly enhanced immunogold signal in the retrieved sections, probably due to the breakage of the covalent bonds between PF and the tissue proteins. Retrieval of the HPF-FS stabilized sections did not increase the immunogold signal, extending the experience by others [160-163]. Interestingly, we observed that retrieval by heating altered the tissue distribution pattern of the epitopes in the PF fixed sections compared with the non-retrieved PF sections and the sections stabilized by HPF-FS. Thus, the results indicate that antigen retrieval by heating may distort the distribution pattern of the immunogold signal and that retrieval, at least by heating, should be performed with care. And as s a consequence, antigen retrieval was not used in our subsequent studies.

Tissue preparation for electron microscopy

Electron microscopy is very useful for the examination of morphological features and antigen labeling at high resolutions. However, cutting of ultrathin decalcified bone sections suitable for electron microscopic examination requires in most cases pre-sectional embedding in a resin. Acrylic resins have traditionally been preferred for ultrastructural immunolabeling compared to conventional epoxy resins [162, 164, 165] due to their combination of sustained immunity as well as stability. In contrast to epoxy resins forming covalent bonds with the tissue proteins, acrylic resins do not copolymerize with the proteins but rather surround the tissue structures making the antigens more prone to labeling after sectioning as the knife-edge cut between the tissue and the resin. However, an alternate method may increase the immunolabeling even more; thawed cryosectioning [166, 167] introduces only a limited degree of aldehyde fixation to the tissue before embedding in sucrose (to avoid crystal formation) and subsequent freezing in liquid nitrogen. Thus, the minimal tissue interaction increases the success-rate for intracellular immunogold labeling and made us able to do further morphological characteristics of the observed intracellular TRAP+ vesicles in paper III.

Histomorphometric evaluations

Bone histomorphometry was performed to quantify gene and protein expression, enzyme activity as well as number of cells and amount of bone areas in groups subjected to different interventions and to allow comparisons between groups. The analyses were preformed

according to stereological principles [168] using a semiautomatic interactive image analyzer program and the sections were coded for the examiner in all cases. Stereological nomenclature was used in paper I, while the suggested nomenclature from the American Society for Bone and Mineral Research [169] was followed in paper II and III.

Histomorphometry is a powerful tool to quantitate morphological differences in the tissue between differently treated specimens. However, it is important to be aware of pitfalls when interpreting the sampled data. In addition, one of the "axioms" in histological evaluation and histomorphometry is that "your histomorphometry is just as good as your histology" [170] and proper tissue fixation and embedding are therefore highly important: the procedures should be carried out in light of subsequent analyses to ensure equal treatment of the tissue specimen in the different groups subjected to examination. We used histomorphometry to a large extent in our studies both for the quantification of amount or size of bone structures, bone cells and cell compartments (paper I, II) and for the quantification of the amount of antigen presented in different bone areas and cell compartments (paper II). The first is rather uncontroversial as commonly defined structures are measured; however, it is important to be accurate and randomize the sample areas due to stereological and histomorphometric principles. The quantification of antigens by antibody labeling in different compartments and specimens is more controversial. The antigen labeling is confounded by false-positive and false-negative labeling as it is difficult to treat the bone tissue identically in every step during the preparation and several steps may interfere with the available number of epitopes [153]. However, the combination of different methods detecting the same antigen may make up for some of the confounders. In paper II, the distribution of TRAP in bone cells was evaluated using histomorphometry and the results compared between animals with different bone phenotypes. Several methods including *in situ* hybridization, immunofluorescence and fluorescence microscopy as well as immunogold labeling and electron microscopy were performed on the same type of bone tissue in order to minimize the effect of false-positive and false-negative labeling. Large standard deviations were demonstrated between the groups in the differently applied methods, which may reflect some of the problems discussed above, although biological variations among the animals can also be part of the reason. However, the results taken together favor that osteoblasts and osteocytes in Ovx-D animals express more TRAP than such cells in sham animals making the conclusion more reliable.

Confocal microscopy and co-localization analyses

The observation of co-localization between antigens provides solid support for their interference; however, it is important to notice that co-localization does not directly prove their functional relationship. Visual co-localization is strongly supported by quantitative co-localization analyses comparing the signal in the same pixel location between multi-channel fluorescence images [171] (reviewed in [172]). With immunofluorescence confocal microscopy, one on the advantages is the opportunity to do quantitative co-localization analyses between differently labeled antigens using semi-automatic software specially designed for such analyses. However, the tissue sections and the images have to be treated properly in order to get reliable results from the co-localization analyses. A confocal microscope improves the possibility for quantitative co-localization analyses as the lasers scan the tissue labeled with different fluorochromes with narrow wave length using the optimized emission filters. In addition, the lasers scan sequentially through the chosen area one-by-one, and together with the narrow wave length, this approach minimizes the bleed-through of the different fluorochromes between the channels. In order to be sure that there really is true co-localization in a three-dimensional (3D) way and not only a coincidental two-dimensional (2D) overlap, z-stacks should be performed allowing images to be obtained through the tissue section. In this way it is possible to perform the co-localization analyses in 3D along the *x, y* and *z*-axis. The results of the analyses and the calculations may be presented as different coefficients; Pearson's correlation coefficient (PCC) is among these and is commonly used to determine co-localization as it measure the strength of a linear relationship between 2 variables [171], (reviewed in [172]). In paper III semi-automatic quantitative co-localization analyses were performed on 2D images using Coloc2 (Fiji, ImageJ) and the results were presented as PCCs. In addition, z-stacks were obtained of the actual cells and tissue sections demonstrating visual 3D co-localization between the different antigens.

5.1.6 Statistics

The statistical analyses were performed in SPSS 12.01 (I) and PASW Statistics 18 (III) for Windows (Microsoft Corporation). Although, the analyses of the mechanical data in paper I differed slightly from a true Gaussian distribution, a two-tailed independent-sample t-test was found suitable for the analyses after discussion with an independent statistician (Betina Kulle, Department of Biostatistics, University of Oslo, Norway, personal communication). In paper II we chose nonparametric tests for *two* and *k* variables (Mann-Whitney and Kruskali-Wallis, respectively) for the semi-quantitative analyses, as they are less likely to have a Gaussian

distribution and to give maximum protection against type I errors. For the measured data we chose parametric tests; Student's *t*-test for *two* variables and one-way analyses of variance (ANOVA) for *k* variables, as measured data are more likely to have a Gaussian distribution. All results were given as means and standard deviations (SD) (paper I, II) with statistical p-values and sample sizes. A p-value of ≤ 0.05 was considered significant. In paper III Pearson's correlation coefficient (PCC) above threshold was calculated using Coloc2 (Fiji, ImageJ) to determine the co-localization of 2 different antibodies. Positive co-localization was considered with PCC ≥ 0.8.

5.2 Discussion of main results

5.2.1 Paper I

The experimental osteoporosis model in female rats was successfully accomplished with undetectable serum levels of 25(OH)D in 94% of the Ovx-D rats and below the human defined deficiency level in all rats [173], in addition to estradiol levels below the human postmenopausal state. The Ovx-D rats developed an osteopenic phenotype with significantly reduced BMD in bone areas with a high content of cancellous bone; however, no cortical bone loss (i.e. increased medullary cavity) was observed. This may be due to the relatively short time-span after ovariectomy (~70 days) [130] and is in line with the observed pattern in the classic ovariectomized (OVX) rat model [146]. In addition, except for significantly decreased bending stiffness in the Ovx-D group, no impaired biomechanical properties were detected in the femur neck or long bone diaphyses. Thus we failed to reproduce the weakening of the femur neck as previously reported in this model [124]. However, based on our results and sample size it is unlikely that an undetected but clinically relevant difference in mechanical strength is present in the femoral neck in the Ovx-D rats at the tested time-point.

Experimental osteoporosis and the mechanical properties of the callus

Old osteoporotic ladies heal from a femur shaft fracture more slowly than young men [174], however, whether this is due to the osteoporosis or the age is not answered by Nikolaou and co-workers. Investigating the fracture healing capacity in osteoporotic humans is difficult and there are still no reports on the fracture healing capacity in osteoporotic men or women compared to sex and age-matched, healthy controls. On the other hand, several experimental studies on animals have been performed over the last 15 years using the classic OVX model

in the rat, with or without dietary interventions [62, 133, 175-189], trying to address the question; does osteoporosis delay fracture healing? However, the results conflict in BMD and mechanical properties of the fracture callus both at early and late healing (defined as < 6 weeks and > 6 weeks, respectively) regardless of the age of the animals at the fracture time-point. Studies which demonstrated decreased healing capacity in the OVX group [133, 175-177, 181, 188] also reported symptoms of delayed healing at the histological level with architectural distortion, decreased mineralization and increased osteoclast number [175-177, 181]. Extended examination of the fracture calluses from the animals in paper I by Melhus and co-workers [62] showed no such difference according to the well known bone remodeling markers OPN, BSP, TRAP and cathepsin K as well as the osteoblast and osteoclast number. However, they found less connective and fibrous tissue and more cartilage and woven bone at 3 weeks of healing in the Ovx-D animals compared to sham. Similar results have also been reported by others and might indicate an impact of osteoporosis on endochondral bone formation in the early period of fracture healing [177, 181, 184, 185, 190]. In our model these differences were undetectable after 6 weeks [62].

Vitamin D, bone strength and fracture healing

Interestingly, our data indicate that the additive effect of vitamin D depletion does not dramatically alter the results reported from OVX rats on normal rat chow or OVX rats fed a low calcium diet (LCD) [178, 182, 183, 187]. Vitamin D-deficiency is common among the human female osteoporotic population [191] and insufficient levels of vitamin D have been reported in osteoporotic patients experiencing a hip fracture [87, 132]. In addition, 1,25D administered orally to OVX rats has resulted in increased fracture callus strength and callus remodeling capacity compared to controls both at 6 and 16 weeks post fracture [192]. Thus it is tempting to assume that the opposite would be the case in a vitamin D deficient state; however, such conclusions cannot be drawn. Moreover, a recent study failed to show any difference in the serum levels of 25(OH)D during fracture healing in individuals suffering from a fragility fracture and low BMD vs. age matched controls with fracture and normal BMD, however, no control group without fracture was included [193]. In both groups the serum level of 25(OH)D was in the range of low (<20ng/mL) as defined by experts [173, 191], and it could not be excluded that low serum level of 25(OH)D makes the individual more prone to fracture regardless of BMD.

Vitamin D favors phosphate and calcium absorption from the intestine as well as the reabsorption of phosphate from the kidneys. On the other hand, vitamin D-deficiency is

followed by impaired mineralization of osteoid, which seems to be mostly dependent on the circulating ion levels of calcium and phosphate. Studies have shown that animals with dietary induced vitamin D deficiency do not develop osteomalacia as long as the levels of calcium and/or phosphate are kept within the normal range [194, 195]. This implies that the impact of the dietary induced vitamin D-deficiency in our rats may have been overridden by the normal amount of calcium and phosphate in the diet, thus with little or no impact on the callus and skeletal mineralization.

Limitations

Among the limitations in paper I are the low healing rates, large SDs in mechanical test results as well as the failure to reproduce the weakening of the femoral neck as previously reported. The low healing rate and the high variance in the measured callus strength, indicate lack of success in inducing reproducible fractures with subsequent standardized group-dependent healing as mentioned above. A larger sample size for biomechanical testing may have reduced the impact of this variation; however, fixation of a tibia mid-shaft fracture with an un-locked intramedullary pin is not a stable situation and it is likely that the large SDs observed in the mechanical testing of the callus still would have been present. Furthermore, it is known that immobilization can reduce or delay fracture healing in mice [196]. This is at least in part supported by the left-right comparison in our study showing decreased BMD of the right (fractured) limb in both groups. Thus, the use of another type of osteosynthesis may have been more optimal to secure a stable situation and thereby reduce the impact of immobilization on the fracture callus.

Rodent strains have shown differences in their fracture healing capacity [128] and the chosen rat strain could therefore also be a confounder. Most of the models on fracture repair in OVX rats have been carried out in Sprague-Dawley rats [133, 175, 178, 181-184, 186]. On the other hand, Wistar rats (as we used) are represented in both groups favoring altered [176, 185] or unaltered [179, 187] fracture healing capacity. The strain used in this case is thus a less likely contributor since the failure in reproducing the weakening of the femur neck [124] may have been due to the variance in genetic background of the Wistar rats.

5.2.2 Paper II & III

TRAP in osteoblasts and osteocytes

TRAP is well known as an osteoclast marker and is present in the resorption lacunae as well as in transcytotic intracellular vesicles during active bone resorption [35, 62, 123, 140, 197].

The presence of osteoclastic TRAP in the resorption lacuna makes the enzyme available for interaction with other bone cells. In addition, it is also reported that osteoblasts and osteocytes expressing TRAP are observed in the metaphysis and in cortical bone close to intracortical remodeling sites as well as at the endosteal and periosteal surfaces [110, 113, 116]. These are all bone sites close to or under active remodeling and there have been hypothesized that TRAP in osteoblasts and osteocytes is somehow related to osteoclast activity [110, 113, 114, 116, 118].

The main purpose of the study in paper II was therefore to test whether enhanced osteoclast activity *in vivo* would result in a subsequent increase in TRAP in osteoblasts and osteocytes. The theoretical background for this hypothesis is supported by the *in vitro* observations from Perez-Amodio and co-workers [114, 115]: They reported that osteoblasts-like cells co-cultured with osteoclasts precursors are able to remove TRAP from the medium and subsequently inactivate it. In addition, TRAP has been suggested as one of the factors coupling bone resorption to bone formation [14, 118] as immature osteoblasts, when cultured in the osteoclast lacuna *in vitro,* rapidly differentiate into a mature, bone-forming phenotype. Osteoblast endocytosis may be a central event in both theories.

The effect of ovariectomy and vitamin D depletion

The Ovx-D rats developed osteopenia as discussed above, and we demonstrated increased osteoclast activity in the Ovx-D rats compared with sham, as anticipated. The increase in osteoclast activity may be due to the estrogen withdrawal, as in menopause or after ovariectomy in fertile women. Estrogen deficiency diminishes osteoclast apoptosis by down regulation of MMP3 and cleavage of FASLG [58] in osteoblasts. In addition, removal of the protective anti-oxidative effect of estrogen on osteoblasts and osteocytes is suggested to increase osteoblast and osteocyte apoptosis leading to enhanced RANKL secretion by the same or neighboring cells [19, 20, 56, 198, 199]. It is also suggested that osteocyte apoptosis directs osteoclasts to bone areas in need for resorption [56]. These effects result in increased osteoclast life-span and activity as well as decreased number and bone-forming activity of osteoblast altering the balance between bone resorption and bone formation with a net bone loss as an end result. With normal levels of calcium and phosphate in the diet, adding vitamin D deficiency to the OVX model would probably not affect the mineralization of osteoid. Thus the increased osteoclast activity observed in the Ovx-D rats was most likely due to estrogen deficiency rather than low levels of vitamin D.

The Ovx-D rats demonstrated increased levels of TRAP+ vesicles in osteoblasts and osteocytes vs. sham in cortical bone, increased levels of TRAP enzyme activity in cancellous bone as well as a tendency to increased levels of TRAP enzyme activity in cortical bone (not significant). The observed increase in TRAP+ vesicles could be explained by the endocytosis theory, however, the increase in TRAP enzyme activity is somewhat more difficult to explain; engulfing TRAP for inactivation is not logically linked with up-regulation of the enzyme activity. On the other hand, Perez-Amodio and co-workers also demonstrated increased TRAP enzyme activity in their co-cultured osteoblasts with a subsequent rapid decrease in the activity, suggesting that this might be due to increased activation of an endogenous fraction [114]. In line with this observation, endocytosed and subsequently activated TRAP could play a role as a coupling factor promoting osteoblast maturation and activation as suggested by Sheu and co-workers [118]. This notion is further supported by Gradin and co-workers [112] who demonstrated increased cortical bone formation as well as osteoblast maturation and differentiation in transgenic mice over-expressing TRAP in osteoblasts. However, this theory does not explain why osteocytes engulf TRAP. Moreover, examining the bone sections for TRAP mRNA and monomeric, uncleaved TRAP (mTRAP) displayed a tendency to enhanced levels of these products in osteocytes in the Ovx-D animals, questioning endocytosis as the main mechanism for the observed increase in TRAP+ vesicles and TRAP enzyme activity in the Ovx-D rats. Thus, the results called for a second “osteoclast activation” model to address the question in some more depth.

The effect of low phosphate and vitamin D deficiency rickets and repletion

Nutritionally induced vitamin D-deficiency together with low levels of phosphate causes rickets and/or osteomalacia with well known morphological changes [123, 140]. Repletion of vitamin D and phosphate stimulates proper mineralization of cartilage and osteoid which in turn leads to activation of chondroclasts/osteoclasts. In addition, high doses of vitamin D stimulate RANKL transcription and osteoclastogenesis directly through activation of VDR/RXR receptor complex in growth plate chondrocytes, osteoblasts and osteocytes [47, 48, 200]. Increased osteoclast activity was observed in rats healing from dietary induced low phosphate and vitamin D-deficiency rickets, as expected, with the highest rate of osteoclast activity, expressed by the CTX/TRAP5b ratio, in healing after 72h. Although all of the groups in the experimental rickets model displayed osteocytes and osteoblasts with TRAP+ vesicles in cancellous bone and TRAP enzyme activity in osteocytes in cancellous and cortical bone, no differences were demonstrated between the groups in the experimental rickets model

regarding TRAP in osteoblasts and osteocytes, despite increased osteoclast activity in healing after 72h. TRAP mRNA was not detected in osteocytes in cortical bone in any of the groups, and though mTRAP was present in all groups in both cancellous and cortical bone indicating TRAP synthesis and translation to protein, there were no differences between the groups. Thus, osteoclasts are less likely the main source of TRAP in osteoblasts or osteocytes *in vivo* neither in experimental osteoporosis nor in experimental rickets and the endocytosis theory is not supported by the results in paper II.

TRAP+ vesicles in osteoblast and osteocytes

The morphological distribution of TRAP in osteocytes and osteoblasts is only sparsely described in the literature: Bonucci and co-workers demonstrated TRAP reaction product in vesicular structures in osteoblasts from calcium-depleted rats [111], Yamamoto and Nagai described TRAP reaction product in the different components of the Golgi complex and structures suggested to be secretory lysosomes in osteoblasts [116] and Reinholt and co-workers observed TRAP+ vesicles in osteoblast-like cells using immunogold technique [201]. We examined bone sections from the experimental osteoporosis and rickets models in paper II as well as bone sections from young, growing rats in paper III using transmission electron microscopy (TEM) and observed TRAP in electron dense vesicles with similar features in osteoblasts and osteocytes in both cancellous and cortical bone. The TRAP+ vesicles in osteoblasts and osteocytes do to some extent differ from the intracellular TRAP+ vesicles observed in osteoclasts [140, 201]. However, TRAP+ vesicles postulated to be secretory lysosomes with regulated secretion have been described in osteoclasts [202] and the vesicles presented by van Meel and co-workers [202] do have a similar appearance as the TRAP+ vesicles we observed in osteoblasts and osteocytes. From their morphological appearance may represent late endosomes or secretory lysosomes involved in intracellular transport, cell communication or in local bone resorption. However, we did not find any support for the latter in paper II. This is in line with the results of Qing and co-workers on unloaded bone and it seems like osteocytic bone remodeling is only present under specific conditions with excessive requirements for calcium and phosphate, as in lactation [21]. However, also direct sclerostin treatment on osteocytes *in vitro* has demonstrated increased osteocyte lacunar area and although the study also demonstrated enhanced levels of TRAP and cathepsin K in the osteocytes, only carbonic anhydrase (CA2) was demonstrated to have an direct effect on the osteocytic osteolysis, as the CA2 inhibitor, acetozolamide prevented the increase in the osteocyte lacunar area [203]. This indicate that TRAP may have a role in osteocytic

osteolysis, but do not prove it as inhibition of TRAP or cathepsin K was not tested, and TRAP may also have an additional role in both osteoblasts and osteocytes.

Semi-quantitative calculations demonstrated increased level of TRAP+ vesicles relative to total cytoplasmic area in osteocytes compared to osteoblast in all animals, as well as in cancellous vs. cortical bone in the experimental rickets model. The latter was also observed for TRAP enzyme activity in all groups favoring a role for TRAP in bone remodeling; there is an obvious structural difference between cancellous and cortical bone and cancellous bone appears to be more metabolic active than cortical bone with a higher bone turnover. This might be explained by a greater surface to volume ratio in cancellous vs. cortical bone [204] and the increase in TRAP+ vesicles and enzyme activity in osteoblasts and osteocytes in cancellous bone might therefore be linked to bone turnover, however, the mechanism remains elusive.

Co-localization of TRAP and RANKL in LAMP1+ vesicles

Lysosomal associated membrane protein 1 (LAMP1) is known to be present in membranes of late endosomes and lysosomal compartments protecting the membrane from auto-digestion (reviewed in [205]). Generally, late endosomes and lysosomes are considered to be degrative organelles where lysosomes represent the end-stage on the endocytotic route degrading endocytosed material. Different subclasses of these organelles do however exist and both late endosomes and lysosomes can function as secretory organelles. Specialized multivesicular late endosomes can fuse with the plasma membrane and cause the release of exosomes [206], and secretory lysosomes can secrete their contents in response to external stimuli [207]. Secretory lysosomes and "ordinary" lysosomes share features such as the presence of lysosomal associated membrane proteins and an acidic luminal pH for the function of the acid hydrolases stored in their lumen. Secretory lysosomes are, however, specialized as they in addition to having a degrading function, serve as organelles for storage of newly synthesized secretory proteins and have the ability to fuse with the plasma membrane [208]. Our observation of LAMP1 in the TRAP+ vesicle membrane justifies the vesicular appearance, however, it does not alone allow a direct determination of the nature of the vesicles; however, the co-localization of TRAP with RANKL indicates that the vesicles might be secretory lysosomes. The group of Suzuki has performed excessive research on the intracellular regulation of RANKL in osteoblast and osteocytes [23, 24, 209, 210] and has demonstrated two pathways for RANKL to the cell surface in both osteoblasts and osteocytes *in vitro*; one minor route transporting RANKL directly from the Golgi complex to the cell surface; and one

major route requiring both vacuolar sorting protein 33 homolog A (VPS33A) [209] in addition to OPG binding to RANKL before the complex is sorted to LAMP1+ secretory lysosomes [24]. The major route seems to be the crucial pathway in the regulation of the osteoclastogenesis, as a defect in the traffic regulatory activity of OPG increases the osteoclastogenic ability *in vitro* despite increased numbers of OPG decoy receptors [24]. The release of RANKL to the cell surface from the secretory lysosomes is suggested to be regulated by the binding of RANK at the surface of osteoclast precursors to small amounts of RANKL presented at the osteoblast or osteocyte membrane from the minor pathway. This will in turn activate Rab27a/b which leads to docking and release of RANKL from the secretory lysosomes [23, 210]. Our TEM immunogold analyses indicate that the TRAP+ vesicles with co-labeling of both RANKL and LAMP1 in osteoblasts and osteocytes *in vivo*, are similar to the secretory lysosomes described as storage compartments for RANKL by the group of Suzuki. This notion is further supported by the immunofluorescence co-localization analyses for both TRAP/RANKL and TRAP/OPG in diaphyseal osteocytes which revealed strong co-localization between the antibodies, as OPG has been described as a traffic regulator for RANKL from the Golgi complex to the secretory lysosomes [24]. The data may imply a role for TRAP in the secretory lysosomes, which is not unlikely as TRAP is an acid phosphatase promoting its function in an acidic milieu. However, it cannot be excluded that TRAP is released together with RANKL from the secretory lysosomes promoting its function outside the cells e.g. as a coupling factor between the osteoclastic bone resorption and the osteoblastic bone formation as suggested by Sheu and co-workers [118].

If the vesicles are secretory lysosomes that secrete their content at the cell surface in order to communicate with other cells in e.g. the bone marrow, we would expect them to be small enough to travel along the osteocyte canaliculi. Our observed TRAP-RANKL-LAMP1+ vesicles are 200-500nm in diameter; You and co-workers [211] have measured osteocyte canaliculi in long bone diaphyses from 15 weeks old mice and based on their results we propose that it is at least a theoretical possibility for vesicles in the range of 200-500nm in diameter to travel along the osteocyte extensions in the canaliculi for secretion. This would make the cell-cell communication possible and may explain a way for RANK and membrane-bound RANKL to interact in order to stimulate osteoclastogenesis.

In addition to the demonstration of TRAP+ vesicles in osteoblasts and osteocytes we observed TRAP in what seems to be vesicular structures in hypertrophic chondrocytes. The presence of TRAP in hypertrophic chondrocytes have been reported by others [62], but not extensively followed up. RANKL has previously been demonstrated in hypertrophic

chondrocytes [8, 20, 212] and also reported located to the same celltype and bone level as OPG [212]. We observed RANKL and OPG co-localized with TRAP in hypertrophic chondrocytes, suggesting the same mechanisms for TRAP in association with RANKL and OPG to be operative in hypertrophic chondrocytes as in osteoblasts and osteocytes.

The demonstration of loop-TRAP/mTRAP in the Golgi complex in osteoclasts, but not in osteoblasts and osteocytes may be related to a limited production, as low levels of synthesis are difficult to detect with the immunogold technique. Furthermore, labeling for TRAP was in osteoblasts and osteocytes restricted to LAMP1+ electron dense vesicles, no significant labeling was observed neither along the synthetic pathway (endoplasmatic retriculum and Golgi) nor early in the endocytotic pathway (plasma membrane, early endosomes, multivesicular bodies). This may indicate that the technique is not sensitive enough to detect small amounts of protein along these pathways, and indirectly supports the assumption that the observed labeling of TRAP is localized to secretory lysosomes where newly synthesized protein gets stored and thus concentrated to a degree that allows detection.

Limitations

One of the limitations in paper II is the difference in age, sex and strain in the experimental models included; the experimental OP rats were 25 weeks old female Wistar rats, while the animals in the experimental rickets model were 10 weeks old male Sprague-Dawley rats. However, as the rats were compared within their own model-system and to untreated controls, this was not judged to be a confounder. On the other hand, it may explain some of the differences observed between the animal models as the male rats were less skeletally matured due to age and sex. The controls in the experimental rickets group did not display TRAP+ vesicles in cortical osteocytes which differed from the sham animals in the experimental osteoporosis model. Extrapolating to TRAP what we know for RANKL, the reason might be that the osteoblasts are the most important bone regulator in young individuals, while the osteocytes are the most important source of RANKL and the main orchestrator of bone remodeling in adults [19, 20]. However, this hypothesis remains to be proven.

Another limitation in paper II was the difference in result between TRAP mRNA and mTRAP, most abundant in the experimental rickets model where no TRAP mRNA was detected in the cortical osteocytes, while mTRAP occurred in all groups. This is probably due to the difference in sensitivity between *in situ* hybridization and immunofluorescence microscopy rather than differences between synthesis and translation to protein.

In paper III the use of a limited number of individuals and at a very young age may question the general validity of our results. We do not know if the observed co-localization of TRAP with RANKL and OPG in the LAMP1+ vesicles in hypertrophic chondrocytes, osteoblasts and osteocytes represent a general mechanism in bone remodeling, and this call for further investigation.

6 CONCLUSIONS

The experimental osteoporosis model (Ovx-D) was successfully established in rats, demonstrating an osteopenic state comparable with the classic rat OVX model implicating that the addition of vitamin D deficiency to the model had no major effect. The model failed to demonstrate any difference in the fracture callus after 6 weeks of healing evaluated by BMD and mechanical testing, thus indicating no difference in fracture healing capacity between the Ovx-D and sham rats. However, the model demonstrated increased osteoclast activity with a corresponding enhancement of the amount of TRAP located to intracellular vesicles in osteoblasts and osteocytes in Ovx-D vs. sham. Similar increases in TRAP were not observed in rats with nutritionally induced phosphate and vitamin D-deficiency rickets or healing from it, despite excessive osteoclast activity during healing. Taken together with the tendency to increased TRAP synthesis in cortical osteocytes in the Ovx-D rats, our results indicate an endogenous origin of TRAP in osteoblasts and osteocytes independent of the osteoclast activity. Further examination of the TRAP+ vesicles in osteoblasts and osteocytes in rat bone tissue demonstrated co-localization of TRAP with RANKL in the vesicles with LAMP1 presented in the vesicle membrane. Co-localization of TRAP with RANKL and OPG in vesicular structures in osteocytes and hypertrophic chondrocytes was also observed. We therefore propose that the TRAP+ vesicles may be secretory lysosomes as RANKL and OPG recently have been demonstrated in LAMP1+ secretory lysosomes *in vitro*. This suggestion indicates a new role for TRAP in bone remodeling, e.g. 1) as a regulator of RANKL secretion; 2) as a contributor to the activation and/or direction of osteoclasts to bone areas in need for remodeling; or 3) as a coupling factor promoting osteoblast differentiation released simultaneously with the osteoclast stimulator (Fig. 11).

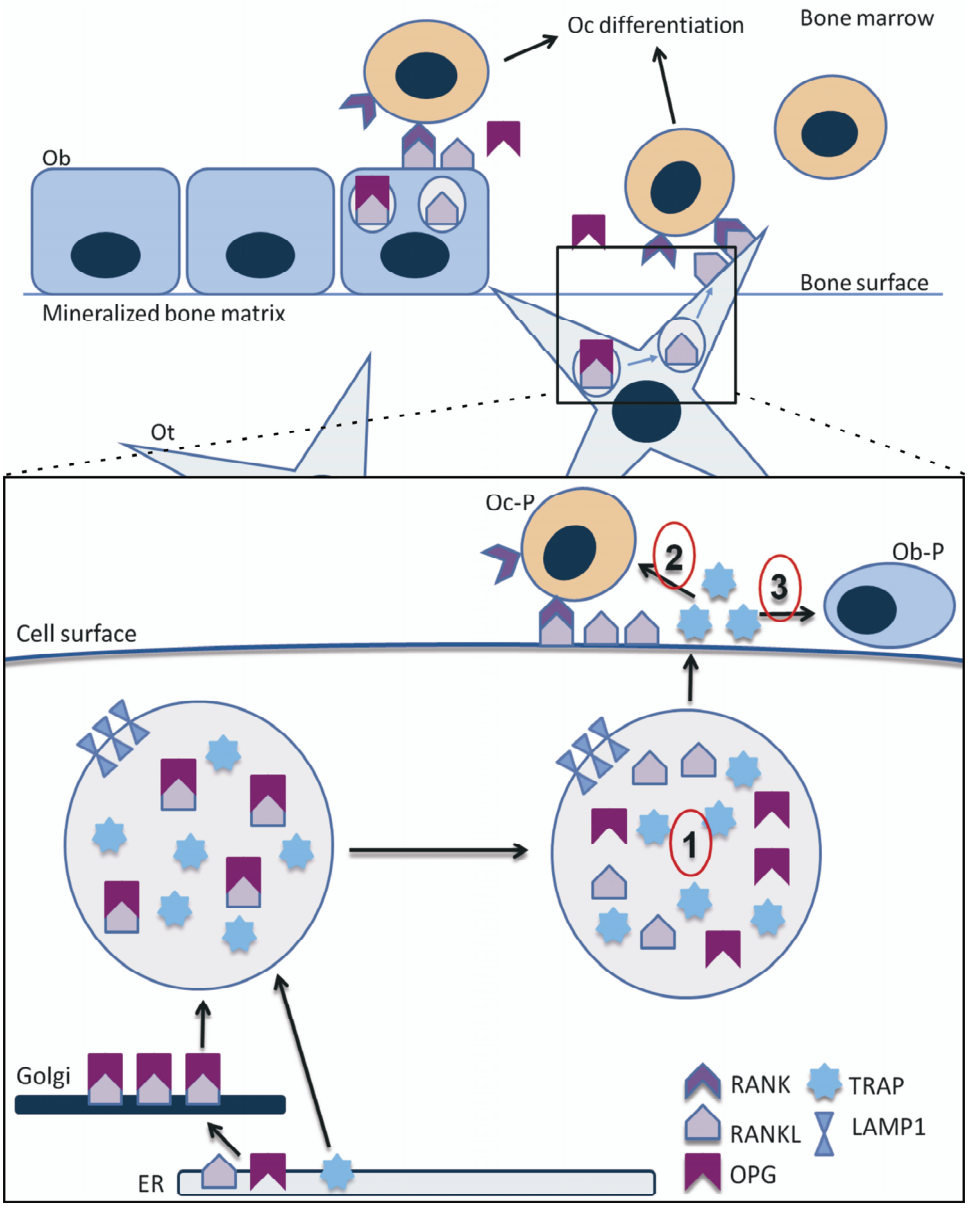


Fig. 11 Schematic overview of the hypotheses RANKL, OPG and TRAP are synthesized in the osteoblast, osteocyte and hypertrophic chondrocytes. OPG binds to RANKL and the complex sorts to LAMP1+ secretory lysosomes (major RANKL secretory route, as suggested in [23, 24]). TRAP co-localizes with RANKL in the LAMP1+ vesicular structures and with OPG at the cellular level and we therefore propose the following hypothetic functions; 1) TRAP interacts with or modifies the RANKL-OPG complex in the secretory lysosomes; 2) TRAP is secreted to the ECM where it serves as a co-stimulatory factor for osteoclast progenitors (Oc-P); or 3) TRAP is secreted to the ECM where it acts as a differentiation and maturation factor for the osteoblast progenitors (Ob-P).

7 FUTURE PERSPECTIVES

7.1 Co-localization of TRAP with RANKL and OPG

Further analyses are needed to elucidate TRAP's role in bone remodeling. The observed results of TRAP/RANKL and TRAP/OPG co-localization in the 3 days old growing rats need to be confirmed in other animal models as well. Observations of the co-localization in other models with different sex, age and strain would strengthen the results. The experimental osteoporosis model and the experimental rickets model are suitable for such studies in addition to their aspects of altered bone metabolism.

It would also be fruitful to study TRAP in relation to RANKL and OPG in cell cultures of osteoblasts and osteocytes by e.g. using the technique described by Uchihashi and co-workers [213]. The advantages of cell culture studies are the opportunity to affect protein synthesis, compartment localization and protein secretion in different ways in order to further elucidate the nature of the TRAP+ vesicles in osteoblasts and osteocytes. Developing osteoblast and osteocytes *in vitro* will hopefully make us able to look into the protein regulation using immunofluorescence and confocal microscopic live imaging techniques in addition to immunogold and TEM of differently treated cells; e.g., immunogold labeling for Rab27a/b [210] as markers for location of TRAP to secretory lysosomes, CD63 as a marker for exocytosis, glucocorticoid treatment to induced cell apoptosis among others.

7.2 The effect of estrogen on osteocyte apoptosis and TRAP expression in osteocytes as well as on the co-localization of TRAP with RANKL and OPG

The OVX rat model may be used with estrogen replacement therapy (HRT) in order to elucidate the effect of estrogens on osteocytes apoptosis in cortical and cancellous bone with focus on TRAP, TRAP/RANKL and TRAP/OPG in these cells.

7.3 The secretion of RANKL from osteoblasts and osteocytes in genetically modulated *in vivo* models

The knowledge on how RANKL from osteoblasts and osteocytes stimulates the osteoclastogenesis still remains elusive. The immunogold technique for TEM on thawed cryosections is a powerful tool in order to look for the vesicles in animal models with altered

RANKL production e.g. to see if the vesicles might represent the main secretory route for RANKL.

7.4 The fracture healing capacity in osteoporosis – a clinical trial with age-matched healthy controls

Animal models mimicking human osteoporosis (OP) seems to be suboptimal to answer the question whether osteoporosis delays fracture healing properly [214]. In addition, the presence of such delay in e.g. the ovariectomized rat does not implicate that this also occurs in humans. A limited number of studies regarding the question have been performed in humans as far as we know [174, 193] with none directly addressing the fracture healing capacity in osteoporotic individuals compared with sex and age-matched controls. We do not know if osteoportic fractures heal more slowly or are more prone to non-unions or implant failure than fractures healing in healthy individuals, however, the latter is assumed as internal fixation of fractures in osteoporic, old individuals is challenging due to their poor bone structure. The large number of fragility fractures as well as the possible costs of pro-healing therapeutics in the future, justifies a clinical study trying to predict the fracture healing capacity in an osteoporotic state. To achieve reliable results, study design as well as well defined semi-non-invasive end-points, are critical and, therefore, a pilot study should be perform.

The following set up is suggested in brief: inclusion of sex and age-matched individuals with similar fracture type/site in need for surgical fixation, age > 40 years (mature skeleton, above peak bone mass), DXA at the time of surgery in order to group the patients (OP/control). OP as defined by WHO [77] (with or without previous fragility fracture). Suggested end-points would be: BMD by DXA or QCT of the callus area at the follow-up consultations; healing of the fracture evaluated by CT; failure of fixation within 12 weeks of healing; non-union after 12 weeks. Follow-ups: 2/3, 6, 12 and 52 weeks post surgery with clinical and radiological examinations (between 12 and 52 weeks control until healing as in ordinary fracture care). Interesting parameters to register at the time of surgery and during the follow-up consultations: estradiol (both sexes at the time of surgery), testosterone and SHBG (in men), 25(OH)D, calcium, phosphate, creatinine, PTH, NSAIDs treatment (should be avoided). If possible; needle biopsies under fluoroscopy of the fracture callus at 2 time-points during healing (e.g. 3 and 6 weeks) in selected patients for histology and gene analyses. Exclusion criteria: < 40 years, anti-osteoporosis therapy (except for vitamin D and calcium), glucocorticoids, anti-convulsives, multitrauma, post-operative infections.

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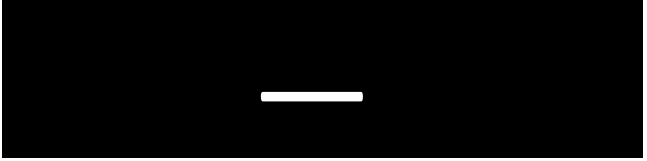
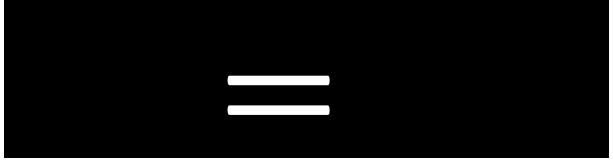
ERRATA

Ref 62 p51 in “General discussion” and ref 17 p3 in paper III are corrected to
Hessle L, Stordalen GA, Wenglén C, Petzold C, Tanner EK, Brorson SH, Baekkevold ES, Önnerfjord P, Reinholt FP, Heinegård D (2013) The skeletal phenotype of chondroadherin deficient mice. PLos One 8:e63080

Ref 104 p65 in “References” is corrected to
Janckila AJ and Yam LT (2009) Biology and clinical significance of tartrate-resistant acid phosphatases: new perspectives on an old enzyme. Calcif Tissue Int 85:465-483





Increased tartrate-resistant acid phosphatase (TRAP) expression in osteoblasts and osteocytes in experimental osteoporosis in rats

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Running title: TRAP in osteoblasts and osteocytes

ABSTRACT

TRAP is known as an osteoclast marker, but osteoblasts and osteocytes in the vicinity of bone remodeling sites also express TRAP. Cell culture studies suggest that osteoblasts endocytose osteoclastic TRAP for inactivation. To evaluate whether changes in osteoclast activity could alter TRAP expression in osteoblasts and/or osteocytes *in vivo,* we studied the ovariectomized and vitamin D-deficient rat (Ovx-D), and rats healing from rickets. Bone sections were analyzed for TRAP gene expression by *in situ* hybridization, TRAP protein by immunogold labeling and TRAP enzyme activity using the fluorescent substrate ELF97. Osteoblasts and osteocytes close to intracortical remodeling sites and bone surfaces demonstrated TRAP, most prominent in cancellous bone and in osteocytes. Intracellular TRAP was located to electron dense vesicles with similar morphology in both cell types. Ovx-D increased osteoclast activity ($p<0.001$) and ELF97+ osteocytes ($p<0.05$) in cancellous bone, but no corresponding increase was observed in the osteocyte lacunar area. The level of TRAP+ vesicles in cortical osteoblasts ($p<0.01$) in Ovx-D was also increased. Enhanced osteoclast activity was noted in healing rickets after 72h ($p<0.05$), but no differences in TRAP expression were detected in osteoblasts or osteocytes. Thus increased osteoclast activity does not affect TRAP expression in osteoblast and osteocytes favoring the notion that increased TRAP in these cells is rather due to increased synthesis. Although the role of TRAP in osteoblasts and osteocytes remains elusive, we speculate that the function is related to the capability of the enzyme to regulate the phosphorylation of proteins known to be expressed by these cells.

Key words: TRAP, Osteoblast, Osteocyte, OVX, Vitamin D

INTRODUCTION

Tartrate-resistant acid phosphatase (TRAP) (ACP5) also known as purple acid phosphatase, uteroferrin or type 5 acid phosphatase [1] has been an established marker for osteoclasts and bone resorption for more than 50 years. TRAP is synthesized as a relatively inactive pro-enzyme (monomeric TRAP (mTRAP)/loop-TRAP/serum TRAP 5a) and proteolytic cleavage by members of the cathepsin family or other proteinases, increases the catalytic activity at least 10-fold [2, 3]. Cleaved, active TRAP is identical to osteoclastic TRAP and serum TRAP 5b[4] and is able to dephosphorylate bone matrix proteins e.g. osteopontin (OPN) and integrin binding sialoprotein (IBSP) [5, 6] as well as to generate reactive oxygen species for bone matrix degradation [7]. Halleen and coworkers have shown that the serum activity of TRAP 5b is significantly elevated in patients with osteoporosis and negatively correlated with bone mineral density (BMD) [8]. Studies on mice have yielded similar results: global deletion of TRAP leads to disturbed endochondral ossification and a mild osteopetrotic phenotype [9, 10], while overexpression of TRAP results in enhanced bone turnover and a mild osteoporotic phenotype [11]. In addition to osteoclasts, TRAP has also been reported in osteoblasts and osteocytes [12-21] closely related to bone surfaces [12, 21] or intracortical remodeling sites [12, 16] in rat bone tissue. The origin and function of TRAP in these cells have been debated; one hypothesis is that osteoclastic TRAP from the resorption lacunae is endocytosed by the osteoblasts and/or osteocytes. This theory is supported by cell culture studies reporting that osteoblast-like cells are able to engulf osteoclastic TRAP and inactivate the enzyme, suggesting that this could control the enzyme activity and prevent further degradation of matrix constituents [17, 18]. However, endogenous TRAP synthesis has been demonstrated in osteoblasts [21] and in osteocytes [16] in areas close to bone resorbing osteoclast, suggesting that TRAP may take part in mechanisms controlling the direction of osteoclastic bone resorption [16]. Qing and co-workers [19] have demonstrated enlarged osteocyte

lacunae and canaliculi and increased amount of TRAP and Cathepsin K in osteocytes in lactating mice, suggesting that osteocytes are able to remodel their own matrix environment through osteoclast-like mechanisms under specific conditions.

To increase the knowledge of TRAP in osteoblasts and osteocytes, we analyzed two experimental rat models with disturbed bone metabolism to investigate whether changes in osteoclast activity could alter TRAP protein expression and enzyme activity in osteoblasts and/or osteocytes *in vivo*. The ovariectomized and vitamin D-depleted rat (Ovx-D) mimics human osteoporosis as seen in the elderly postmenopausal women with reduced BMD of metaphyseal bone [22]. Osteoclast activity and TRAP protein expression in osteoblasts and TRAP activity in osteocytes were increased in Ovx-D rats. Moreover, Ovx-D rats presented a tendency to increased TRAP mRNA expression in osteocytes, questioning the hypothesis of endocytosis being the mechanism enhancing TRAP protein expression and enzyme activity in osteoblasts and osteocytes in these rats. To further address this question, rats healing from nutritionally induced low phosphate and vitamin D-deficiency rickets (experimental rickets) were analyzed as a model of increased osteoclast activity [23]. However, such rats did not show any differences in the level of TRAP protein expression or enzyme activity neither in osteoblasts nor in osteocytes, making it less likely that osteoblasts and osteocytes endocytose osteoclastic TRAP.

MATERIALS AND METHODS

All analyses were preformed on coded sections using AnalySIS FIVE (Olympus Soft Imaging Solutions GmbH, Münster, Germany) following the suggestions for standardized nomenclature from ASBMR [24].

Animal experiments

Guide for the Care and Use of Laboratory Animals [25] was followed and the study protocols approved by the Norwegian National Animal Research Authority. The Ovx-D model has been reported in detail previously [22]. Low phosphate and vitamin D-deficiency rickets and healing for 48h and 72h were induced as described by Hollberg et al 2005 [23]. Blood was sampled from the animals and their tissues fixed by vascular perfusion [22] at the end of the experiments.

Tissue preparation

Femurs and tibias were dissected free, immersed in 2% phosphate-buffered paraformaldehyde and decalcified in 7% EDTA with 0.5% paraformaldehyde for 40 days. Bone tissues for light microscopy or fluorescence microscopy were paraffin-embedded and 2-3µm thick sections were cut, picked up on glass-slides and rehydrated in series of graded alcohols. Bone tissues for transmission electron microscopy (TEM) were cut into small samples (~1 mm^3), fixed in 2% paraformaldehyde and 0.5% glutaraldehyde and embedded with progressive lowering of temperature (Leica EM AFS, Leica Microsystems AG, Wetzlar, Germany) in the acrylate- and methacrylate-based resin Lowicryl HM23 according to our established protocol [26]. Ultrathin sections (75 nm) were mounted on formvar-coated nickel slot grids.

Osteoclast activity

Total number of osteoclasts relative to tissue volume (N.Oc/TV) and osteoclasts surface relative to bone surface (Oc.S/BS) were estimated by point counting in a squared grid within 500µm from EMB at TEM micrographs. An osteoclast was defined as a multinuclear cell attached to bone surface or in the intertrabecular space with characteristics such as ruffled border, intracytoplasmic vesicles and abundant mitochondrial profiles. Twenty micrographs

from each animal were analyzed and the ratio (Oc.S/BS)/(N.Oc/TV) calculated for each animal and compared between Ovx-D and sham as a parameter of osteoclast activity [27]. In the experimental rickets group, commercially available kits were used for determination of osteoclast-derived C-telopeptide fragments of collagen type I (CTX) (RatLaps™ EIA, Immunodiagnostic Systems Ltd., Tyne & Wear, UK) and osteoclast derived TRAP 5b (RatTRAP™ Assay, Immunodiagnostic Systems Ltd., Tyne & Wear, UK). Serum was analyzed in all animals and the results of the CTX/TRAP 5b ratio, as a parameter for osteoclast activity [27], calculated for each animal and compared between the groups.

In situ hybridization

TRAP gene expression was studied by *in situ* hybridization. A gene sequence for rat TRAP [28] was amplified by conventional PCR using cDNA from rat bone and oligonucleotide forward and reverse primers; rnTRAP.for 5'-ACGCCAATGACAAGAGGT TC-3', rnTRAP.rev 5'-ACATAGCCCACACCGTTCTC-3'(Life Technologies Co., Carlsbad, CA, USA) and cloned in a Dual Promoter TA Cloning Kit (Life Technologies Co., Carlsbad, CA, USA). The cloned insert was sequenced to establish the orientation (Seqlab, Göttingen, Germany). A digoxigenin (DIG)-conjugated complementary RNA probe was synthesized using T7 or Sp6 polymerase to yield the probe in the sense or antisense direction (DIG-labeling kit, Roche Diagnostics AS, Oslo, Norway). Longitudinal sections from tibia diaphysis (Ovx-D/sham) and femur diaphysis (experimental rickets) were subjected to hybridization following our established protocol [29]. TRAP mRNA+ osteocytes were quantified in cortical bone within 4-10 mm from the proximal epiphyseal/metaphyseal border (EBM) by point counting in a squared grid. Three sections were examined from each animal and their means compared between the groups. The tibia diaphyses were examined twice with interclass correlation of $p<0.001$, and Cronbach's alfa of 0.94. Staining of osteoclasts from

femur metaphysis in healing for 72h were used as positive control. The sense probe did not show any staining.

Immunofluorescence

To estimate TRAP enzyme activity and the putative co-localization of the translated monomeric TRAP protein (mTRAP) and the enzyme activity, fluorescence based staining with rabbit anti-mTRAP and ELF97 was performed. With low pH (<6.0) ELF97 is cleaved by activated acid phosphatase yielding a bright yellow-green-fluorescence precipitate [30, 31]. Rabbit anti-mTRAP was the same as previously used [32]. ELF97 Endogenous Phosphatase Detection kit, AlexaFlour555 conjugated secondary antibody and DAPI Nucleic Acid Stain were purchased from Molecular Probes (Invitrogen Co., Eugene, OR, USA). Longitudinal sections from distal femur metaphysis and diaphysis were analyzed. Images were obtained by Nikon DS-Fi2 color camera (Nikon Instruments Inc., Melville, NY, USA) using UV and Cy3 filters and added in ImageJ [33]. ELF97+ osteocytes (Ot), mTRAP+ Ot, ELF97mTRAP+ Ot and total Ot were quantified in cancellous bone within 1 mm into the metaphysis from the proximal EMB and in cortical bone within 4-10 mm from the proximal EMB. The means were calculated for each animal with respect to the parameters above and used for comparison between the groups. Non-specific rabbit IgG served as negative control for mTRAP, while TRAP enzyme activity was inactivated using 100μM molybdate before adding ELF97 to evaluate the background fluorescence.

Immunogold labeling for TEM

To evaluate the distribution of TRAP in osteoblasts and osteocytes, bone sections from tibia diaphysis (Ovx-D/sham) and proximal tibia metaphysis and diaphysis (experimental rickets) were labeled with rabbit anti-TRAP (SB-TR103, Immunodiagnostic Systems Ltd., Tyne &

Wear, UK). The immunogold labeling was performed as earlier described [34]. Non-specific rabbit-IgG served as negative control. Micrographs of 10-20 osteoblasts; defined as mononuclear cells attached to osteoid or bone matrix with prominent ER and Golgi complexes, and osteocytes; defined as mononuclear cells embedded in bone matrix with characteristic canaliculi, were randomly sampled from each animal. TRAP+ vesicle was defined as a vesicle of moderate electron density containing $\geq$4 gold particles. Area of TRAP+ vesicles (TRAPv.Ar) relative to the area of cytoplasm (Cy.Ar) was measured in each osteoblast and osteocyte and the mean of the ratios (TRAPv.Ar/Cy.Ar) for each animal compared between the groups. The cells were analyzed twice with respect to TRAPv.Ar/Cy.Ar with interclass correlation of $p<0.001$, and Cronbach's alfa of 0.98. Large standard deviations (SDs) in the ratios were observed for both osteoblasts and osteocytes within the Ovx-D and sham. To elucidate whether this phenomenon was due to differences between the animals in each group or in each animal, 8 bone levels in one animal from each group were examined. The results displayed that the large SDs between the animals corresponded to the SDs between bone levels in each animal, indicating large biological variation (data not shown).

Osteocyte lacunar area

Longitudinal tibia mid-diaphyseal sections from Ovx-D and sham at the same bone level were subjected to conventional hematoxylin-eosin-saffron (HES) staining. The osteocyte lacunar area was measured within 1 mm at 3 discrete sites separated by 1 mm in a cross-sectional manner. Both cortices were included and 200-250 osteocytes were measured per animal. The means of the osteocyte lacunar area were calculated and compared between the groups.

Statistics

The statistical analyses were performed in PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). Parametric tests were used to compare the measured data; Student's *t*-test for *two* variables and one-way analyses of variance (ANOVA) for *k* variables. Nonparametric tests; Mann-Whitney for *two* variables and Kruskal-Wallis for *k* variables, were applied on the semi-quantitative data. A *p*-value of 0.05 was considered significant in all tests.

RESULTS

Animal models

The Ovx-D rats developed osteoporosis with reduced trabecular bone volume (BV/TV) in the femur head, $p<0.001$, and decreased BMD in the femur neck and the lower lumbar vertebra, $p<0.001$, (Online Resource 1a, b) as well as undetectable serum levels of 25(OH)D and serum estradiol within the human postmenopausal range [22]. Low phosphate and vitamin D deficiency induced rickets with characteristic morphological changes (Online Resource 1c) were in line with previous experience with the model [23].

Enhanced osteoclast activity in the animal models

To be able to evaluate TRAP positivity in osteocytes and osteoblasts in relation to osteoclast activity, we calculated osteoclast activity as Oc.S/BS relative to N.Oc/TV and CTX relative to TRAP 5b in serum in Ovx-D/sham and experimental rickets, respectively. Increased osteoclast activity was observed in Ovx-D vs. sham (Fig. 1a), as well as in healing rickets after 72h compared to fulminant rickets and normal controls reflecting the healing of the growth plate with enhanced resorption monitored by an increased CTX/TRAP 5b ratio in serum (Fig. 1b).

TRAP is increased in osteoblasts and osteocytes in Ovx-D

In cancellous bone ELF97+Ot/Ot and co-localized ELF97mTRAP+Ot/Ot were increased in Ovx-D vs. sham (Fig. 2, Fig. 3a). Ovx-D also demonstrated a tendency to increase in ELF97+Ot/Ot and ELF97mTRAP+Ot/Ot vs. sham in cortical bone (Online Resource 2, Fig. 3b). TEM analyses showed TRAP in intracellular electron dense vesicles (200-500nm in diameter) with similar morphological features in both osteoblasts and osteocytes in cortical bone. However, no general pattern of location in the cytoplasm was detected and we were not able to demonstrate any fusion between TRAP+ vesicles and the cell-membrane, or any coated pits at the cell-surface containing TRAP (Fig. 4a-h). Semi-quantitative measurements of the area of TRAP+ vesicles relative to total cytoplasmic area (TRAPv.Ar/Cy.Ar) showed an increased ratio in osteoblasts and osteocytes in Ovx-D compared with sham, significant in osteoblasts (Fig. 4i). *In situ* hybridization demonstrated TRAP mRNA in osteocytes in cortical bone close to intracortical remodeling sites and bone surfaces in both Ovx-D and sham (Fig. 5a-c), however, only a small proportion of the osteocytes in cortical bone were TRAP mRNA+: 2.9% in Ovx-D vs. 0.09% in sham. Although the difference appeared striking, the result was not statistically significant (Fig. 5e).

Healing from rickets does not alter TRAP in osteoblasts or osteocytes

No difference was detected in TRAP enzyme activity (ELF97+Ot/Ot), mTRAP+Ot/Ot or ELF97mTRAP+Ot/Ot in neither cancellous nor cortical bone in any of the groups in the experimental rickets model (Fig. 6). The TRAP+ vesicles were observed in both osteoblasts and osteocytes in cancellous bone and presented similar features as in osteoblasts and osteocytes in the experimental osteoporosis model. However, there was no difference in the ratio TRAPv.Ar/Cy.Ar between the groups. In cortical bone only a few TRAP+ vesicles were observed in osteocytes and none in osteoblasts. TRAP mRNA *in situ* hybridization in femur

diaphysis failed to demonstrate TRAP mRNA+ osteocytes in any of the groups, despite staining in the metaphyseal osteoclasts used as positive controls (data not shown) and with no staining for the sense probe.

TRAP+ vesicles are more abundant in osteocytes compared with osteoblasts

TEM revealed increased TRAPv.Ar/Cy.Ar ratio in osteocytes vs. osteoblasts in cancellous bone for the animals in the experimental rickets model, $p<0.05$ (rickets and controls), $p<0.01$ (healing after 48h), (Online Resource 3a). In cortical bone osteocytes from the Ovx-D and sham rats also demonstrated increased TRAPv.Ar/Cy.Ar ratio vs. osteoblasts, significant in sham, $p<0.001$ (Online Resource 3b). Only a small number of TRAP+ vesicles were detected in cortical osteocytes and none in cortical osteoblasts in the experimental rickets model.

TRAP activity in osteoblasts and osteocytes is most prominent in cancellous bone

TRAP enzyme activity in osteocytes (ELF97+Ot/Ot) was increased in cancellous vs. cortical bone in all groups from both animal models, $p<0.01$ (Ovx-D, sham, rickets, healing after 48h and controls), $p<0.05$ (healing after 72h), (Online Resource 4a). The vesicle ratio TRAPv.Ar/Cy.Ar was increased in cancellous vs. cortical bone in osteoblasts and osteocytes in all animals in the experimental rickets groups; Ot: $p<0.001$ (rickets and healing after 48h), $p<0.05$ (healing after 72h), $p<0.01$ (controls); Ob: $p<0.001$ (rickets, healing after 48h and controls), $p<0.01$ (healing after 72h) (Online Resource 4b, c).

No difference in osteocyte lacunar area in cortical bone in Ovx-D vs. sham

To elucidate whether the increased level of TRAP in osteocytes in Ovx-D could be related to increased local resorption as described for lactating mice [19], osteocyte lacunar area in cortical bone was measured, but no difference was detected between the groups (Fig. 7).

DISCUSSION

Osteoclastic TRAP has been demonstrated in transcytotic intracellular vesicles as well as in the ruffled border beneath the osteoclast during active bone resorption [7, 23, 29, 35, 36]. The secretion of TRAP from the osteoclast to the resorption lacuna makes TRAP available for other bone cells and TRAP has been suggested as one of the "coupling-factors" between bone resorption and bone formation [37]. Previous studies have demonstrated TRAP in osteoblasts and osteocytes in areas close to active bone resorption sites [12, 16, 21], which has led to the hypothesis that osteoblasts and/or osteocytes engulf osteoclastic TRAP for inactivation [17]. We tested this hypothesis *in vivo* by analyzing TRAP expression in osteoblasts and osteocytes in two animal models with increased osteoclast activity; the experimental osteoporotic rat and rats healing from dietary induced rickets. In both models osteocytes and osteoblasts in cancellous bone and in cortical bone close to intracortical remodeling sites and endosteal/periosteal surfaces demonstrated TRAP gene expression and translation to protein as well as TRAP enzyme activity, which are in line with former observations. TEM analyzes revealed TRAP in intracellular vesicles with identical morphological features in osteoblasts and osteocytes in all the animals in both experimental models. This observation indicate that TRAP in osteoblasts and osteocytes might be located to endosomes, involved in intracellular transport or stored in vesicular compartments for secretion. Moreover, the Ovx-D group demonstrated an increased ratio of TRAP+ vesicles in osteoblasts in cortical bone and increased TRAP enzyme activity in osteocytes in cancellous bone, and we also observed a tendency to enhanced levels of TRAP gene expression in osteocytes in cortical bone. These results indicate that the observed increase in TRAP protein expression and enzyme activity in osteoblasts and osteocytes is due to increased synthesis and not increased osteoclast activity. Moreover, no changes were demonstrated between the animals in the different groups in the experimental rickets model regarding TRAP in osteoblasts and osteocytes, despite the

increased osteoclast activity in rats healing for 72h. Thus, enhanced osteoclast activity does not change TRAP expression *in vivo* in osteoblasts or osteocytes in our models. Consequently, TRAP observed in osteoblasts and osteocytes is not engulfed osteoclastic TRAP but rather synthesized in the respective cells. The theory of osteoblast and osteocyte endocytosis of osteoclastic TRAP[17] is therefore not supported by our results. A similar conclusion has been drawn by Bonucci and co-workers [13]: they observed that the increased level of TRAP positive osteoblasts in calcium depleted rats returned to normal when calcium was repleted despite unchanged levels of TRAP positive osteoclasts.

Comparison of TRAP expression in cancellous vs. cortical bone demonstrated enhanced levels of TRAP enzyme activity in osteocytes in all animal groups as well as an increase in the ratio of TRAP+ vesicles in osteoblasts and osteocytes in the animals in the experimental rickets groups. There is an obvious structural difference between cancellous and cortical bone and cancellous bone appears to be more metabolic active than cortical bone with a higher bone turnover. This might be explained by a greater surface to volume ratio in cancellous vs. cortical bone [38]. The increase in TRAP+ vesicles and enzyme activity in osteoblasts and osteocytes in cancellous bone might therefore be linked to bone turnover, however the mechanism remains elusive.

Qing and co-workers observed increased TRAP as well as cathepsin K in osteocytes in lactating mice [19] with a corresponding increase in the osteocyte lacunar area. In a recent study Kogawa and co-workers [39] show that sclerostin increases the expression of TRAP, cathepsin K and carbonic anhydrase (CA2) in osteocytes with a resulting increase in the osteocyte lacunar area. The effect is reversed by the CA2 inhibitor acetozolamide, which indicates that the osteocytic osteolysis is at least partly dependent on CA2 and its response to sclerostin. However, the effects by inhibition of TRAP or cathepsin K on the osteocyte lacunar area were not reported. Taken together, both lactation and sclerostin seem to enhance

TRAP expression in osteocytes as well as the ostocytic osteolysis. We investigated the effect of TRAP on the osteocytic osteolysis in the experimental osteoporosis model, but failed to demonstrate any difference in the osteocyte lacunar area between Ovx-D and sham despite increased osteocytic TRAP in the Ovx-D animals. An explanation for this may be that we did our measurements on decalcified tissue sections. However, this method has been used by others with success [39]. Consequently, we propose that osteocytic TRAP is not solely related to osteocytic osteolysis but has an additional role in osteocytes. Our TEM observations of TRAP located to intracellular vesicles with similar morphological features in both osteoblasts and osteocytes indicate that TRAP may have corresponding functions in the two cell types. Moreover, osteoblasts do not normally dissolve bone mineral and the observed increase in osteoblastic TRAP in the Ovx-D animals is therefore unlikely to be related to local mineral handling by the osteoblasts. In conclusion, the role of TRAP in osteoblasts and osteocytes still remains elusive. However, our results support the notion that TRAP may have another not yet clarified role in osteocytes, in addition to the suggested contribution in local mineral handling. It is proposed that the function of TRAP in osteoblasts and osteocytes involves the capability of the enzyme to regulate phosphorylation of proteins known to be expressed by these cells such as DMP1, MEPE and FGF23.

ACKNOWLEDGEMENTS

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FIGURES

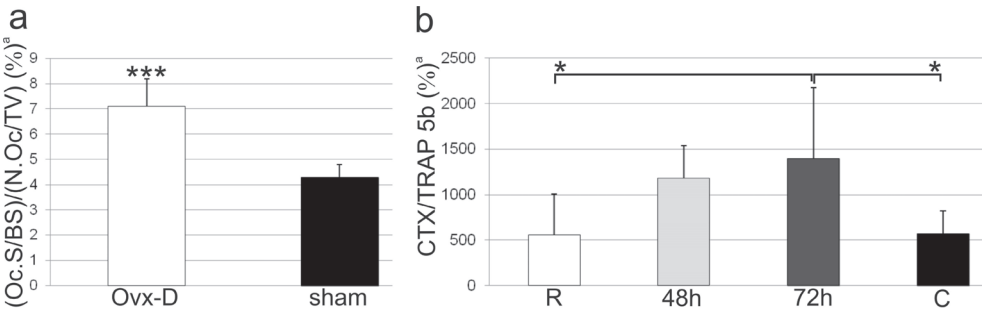


Fig. 1 Osteoclast activity

a The ratio of (Oc.S/BS)/(N.Oc/TV) in cancellous bone was increased in Ovx-D vs. sham (Student's t-test, n=7/7). **b** The ratio of serum CTX/TRAP 5b was increased in healing for 72h compared with fulminant rickets (R) and controls (C) (ANOVA, n=7/7/7/7). [a]The results are presented with mean and SD, * $p<0.05$, *** $p<0.001$

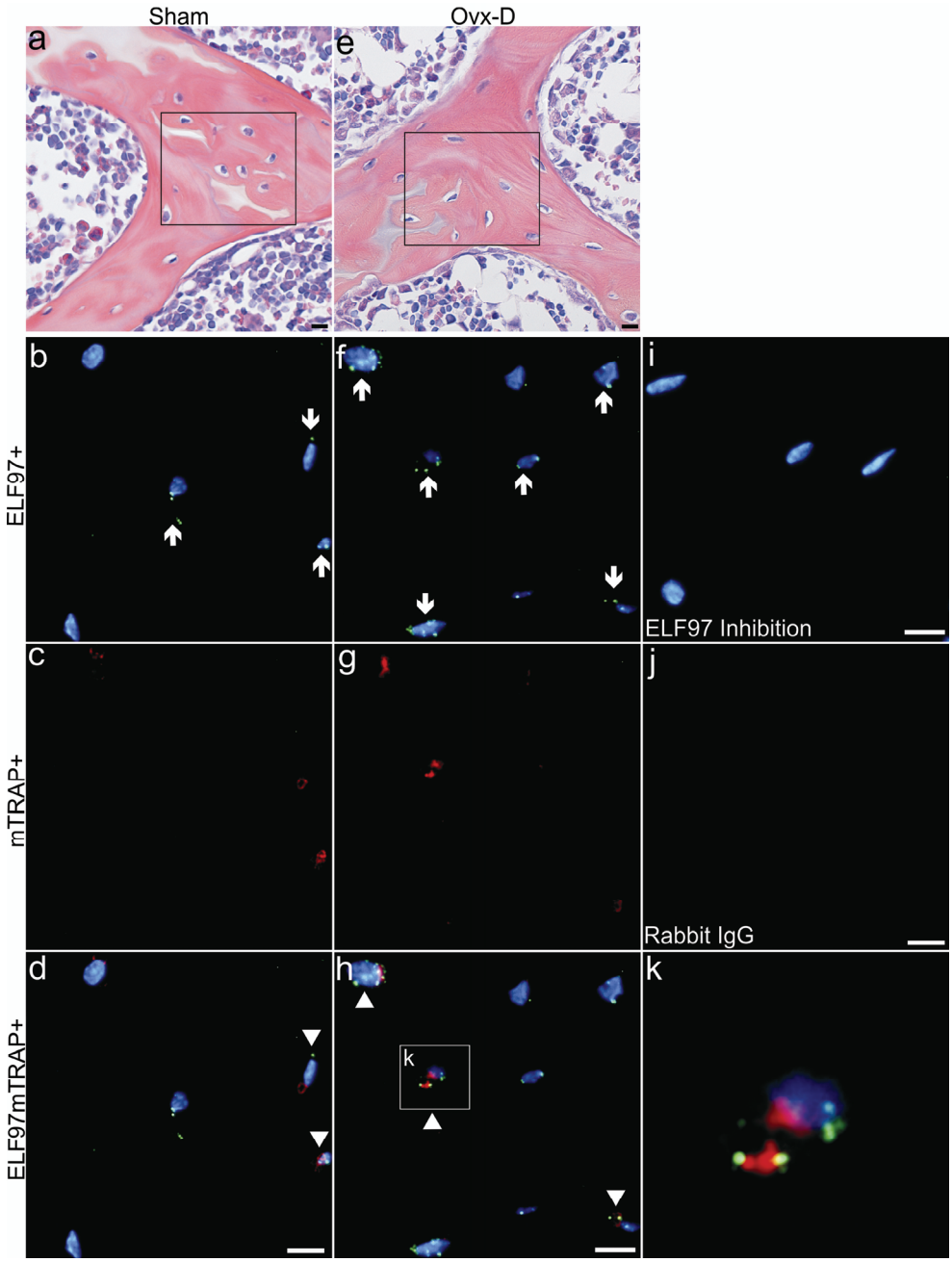


Fig. 2 TRAP enzyme activity and monomeric TRAP (mTRAP) immunolabeling in osteocytes (Ot) in cancellous bone from distal femur metaphysis **a** HES stained sections show the tissue architecture of cancellous bone in sham and **e** Ovx-D. The black outlines demonstrate

corresponding areas to the immunofluorescence images. **b-d** ELF97+ Ot (yellow-green, arrows), mTRAP+ Ot (red) and ELF97mTRAP+ Ot (arrowheads) in sham and **f-h** Ovx-D. **i** Inhibition of TRAP enzyme activity with molybdate demonstrated low background fluorescence for ELF97. **j** Unspecific rabbit IgG served as negative control for mTRAP with low background fluorescence. **k** A high power image shows the staining of ELF97 and mTRAP in the osteocytes. Scalebars 10µm

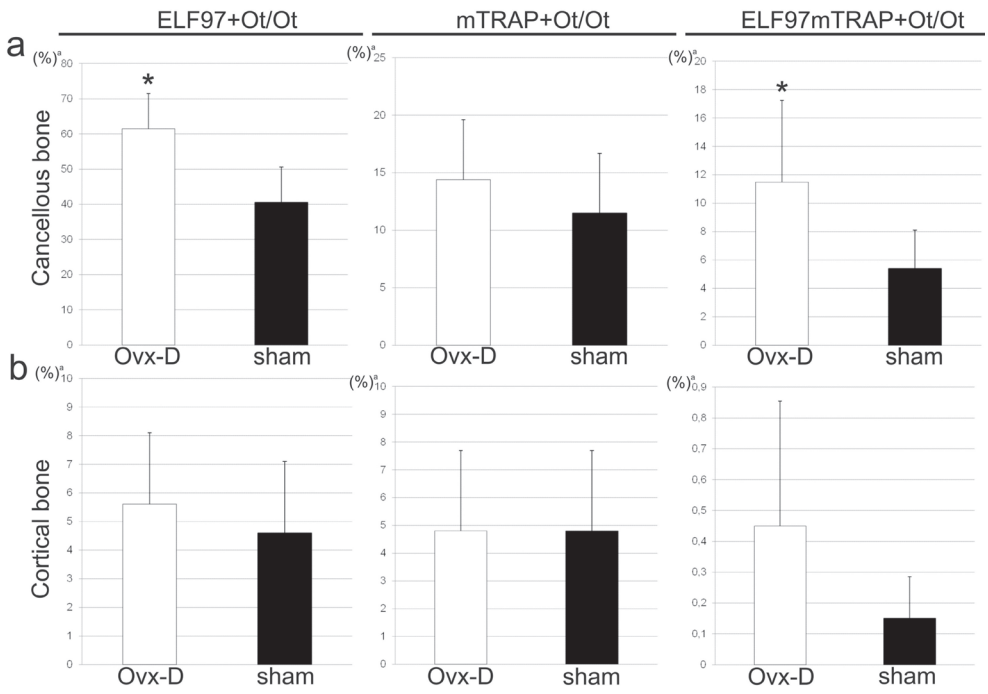


Fig. 3 TRAP enzyme activity in osteocytes in cancellous and cortical bone in the experimental osteoporosis model **a** ELF97+ Ot/Ot and ELF97mTRAP+ Ot/Ot were significantly increased in Ovx-D vs. sham in cancellous bone, **b** while there were no significant differences between the groups in cortical bone (Mann-Whitney test, n=7/7). [a]The results are presented with mean and SD, * $p<0.05$

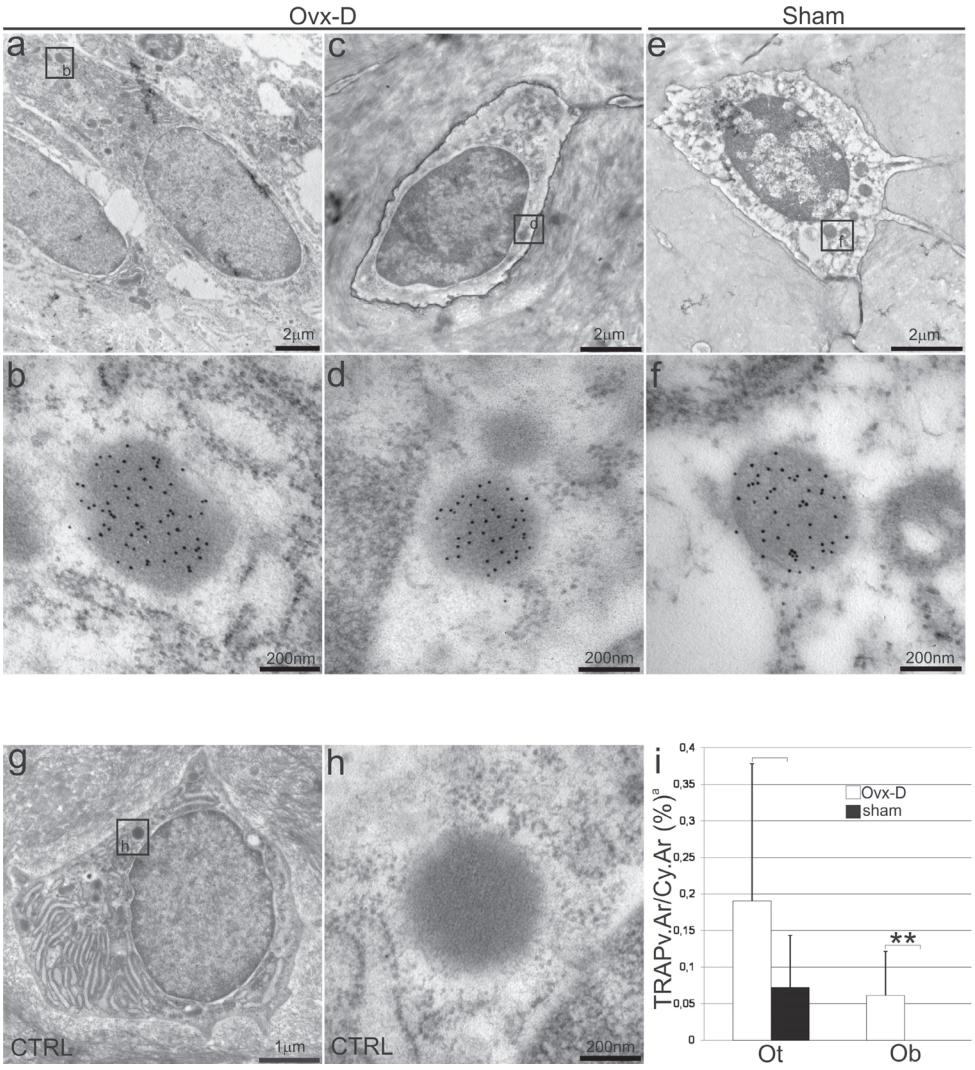


Fig. 4 TEM micrographs from tibia diaphysis in Ovx-D and sham. The images with overview and close-ups show examples of immunogold labeling for TRAP in intracellular vesicles in osteoblasts (Ob) and osteocytes (Ot) in cortical bone; **a, b** Osteocyte and **c, d** osteoblast from Ovx-D and **e, f** osteocyte from sham with TRAP+ vesicles. **g, h** Unspecific rabbit-IgG served as negative control and did not label the vesicles in Ovx-D. **i** Significantly increased TRAP vesicle area vs. area of cytoplasm (TRAPv.Ar/Cy.Ar) ratio in osteoblasts in Ovx-D vs. sham (Mann-Whitney test, n=7/7). [a]The results are presented with mean and SD, ** $p<0.01$

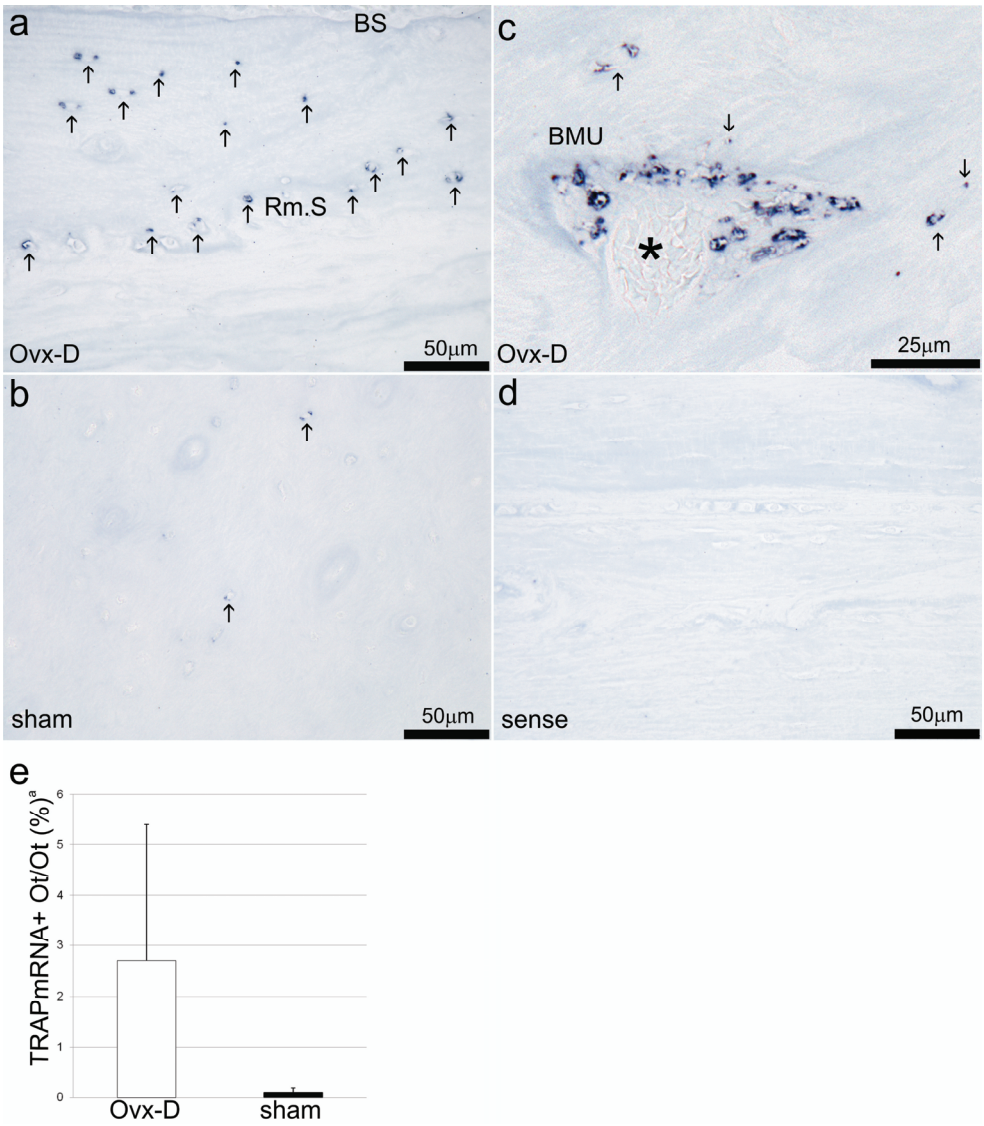


Fig. 5 *In situ* hybridization for TRAP mRNA in tibia diaphysis in Ovx-D and sham animals. **a** TRAP mRNA positive osteocytes (Ot) (arrows) were stained dark blue and observed closely related to the bone surface (BS) and the bone remodeling surface (Rm.S) in Ovx-D. **b** Sham animals demonstrated a limited number of TRAP mRNA positive osteocytes (arrows) **c** The method was confirmed by positive staining of an osteoclast in a bone morphogenic unit (BMU) with TRAP mRNA positive osteocytes (arrows) within close vicinity (Ovx-D). (The

asterisk marks a central capillary.) **d** The sense-probe displayed no staining and served as a negative control. **e** There was no significant difference in TRAPmRNA+Ot/ Ot between Ovx-D and sham (Mann-Whitney test, n=7/7). [a]The results are presented with mean and SD

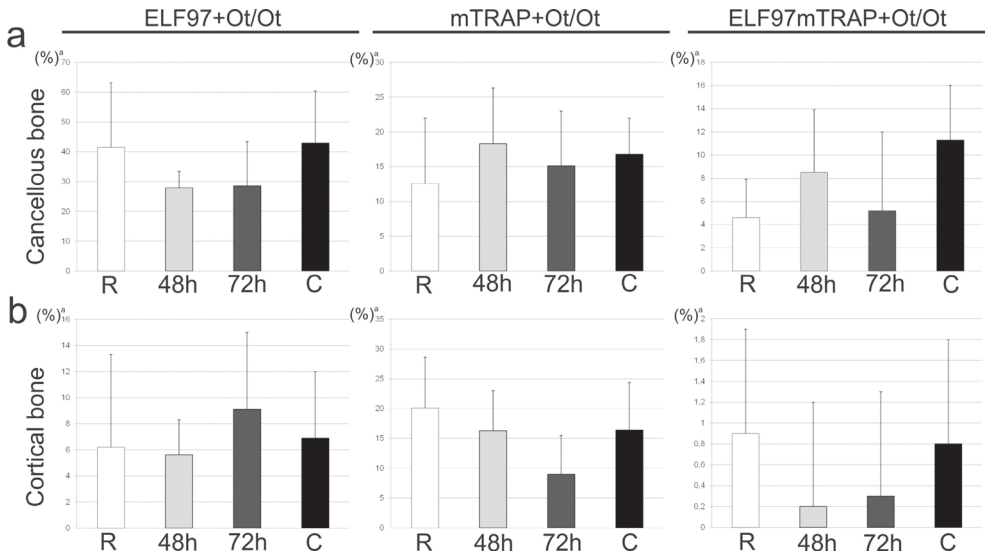


Fig. 6 TRAP enzyme activity in osteocytes (Ot) **a** cancellous and **b** cortical bone in the experimental rickets model demonstrated no significant differences between the groups; fulminant rickets (R), healing for 48h, healing for 72h and controls (C) (Kruskali-Wallis test, n=7/7/7/7). [a]The results are presented with mean and SD

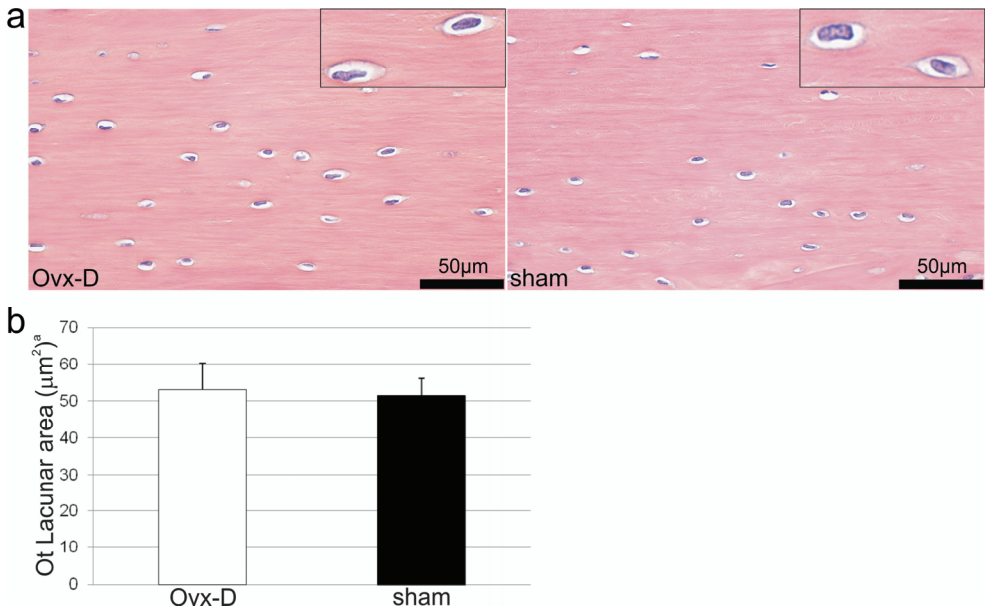
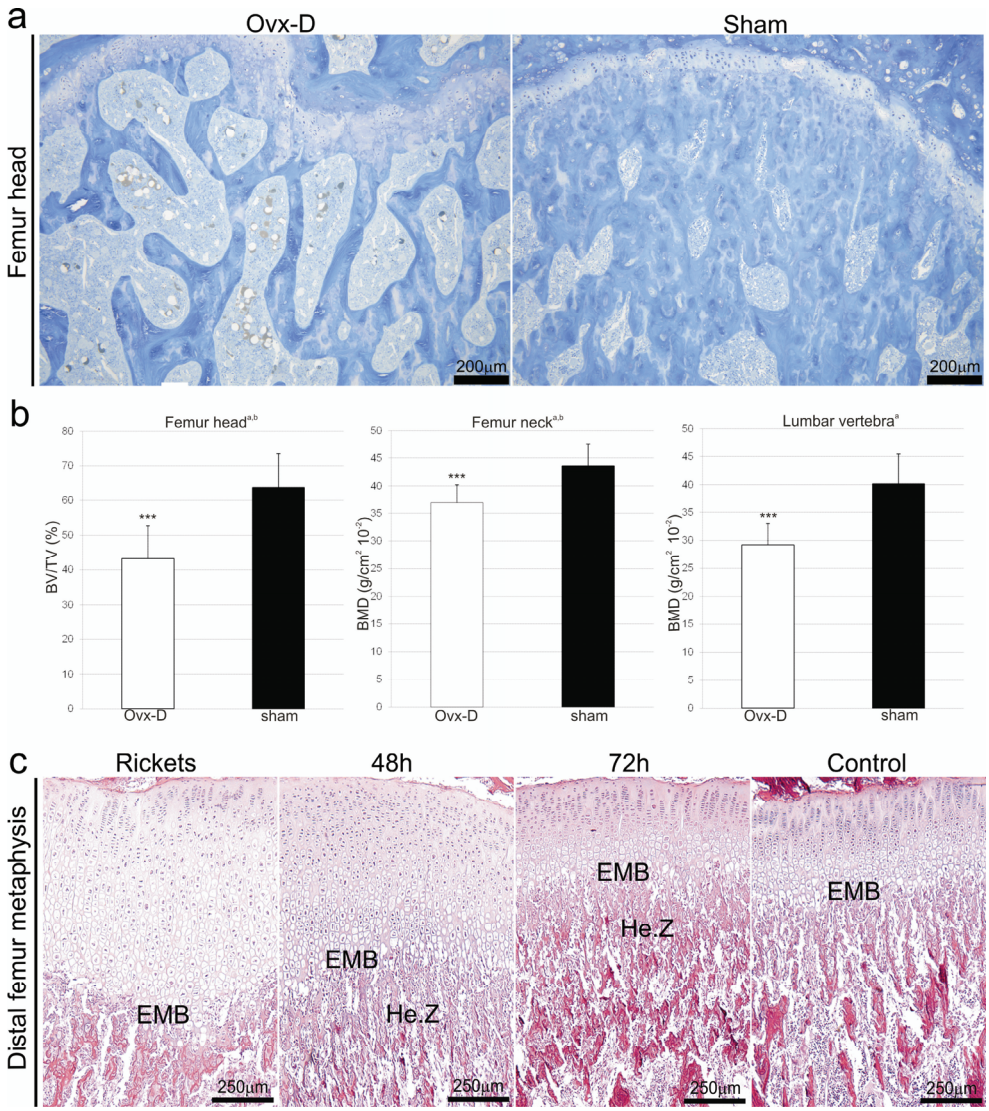


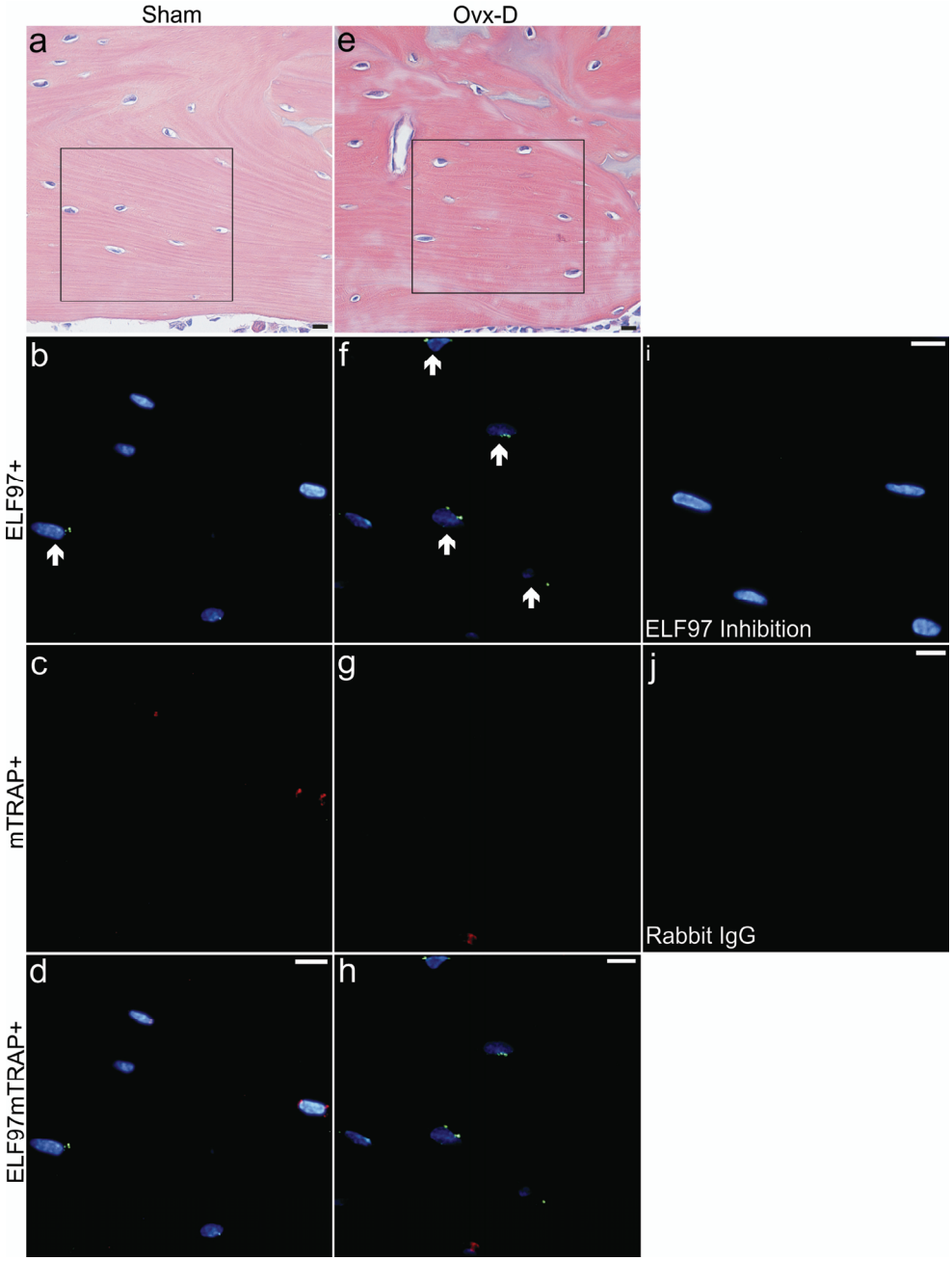
Fig. 7 a Cortical bone sections (tibia diaphyses) from Ovx-D and sham were formalin fixed, paraffin embedded and cut in 3µm thick sections before conventional hematoxylin-eosin-saffron staining was performed. A clear definition of the osteocyte lancunar outline was obtained after staining of the tissue sections for both Ovx-D and sham. This is demonstrated by the high power images inserted. **b** There was no difference in the osteocyte (Ot) lacunar area between Ovx-D and sham (Student's t-test, n=7/7). [a]The results are presented with mean and SD

ONLINE RESOURCES



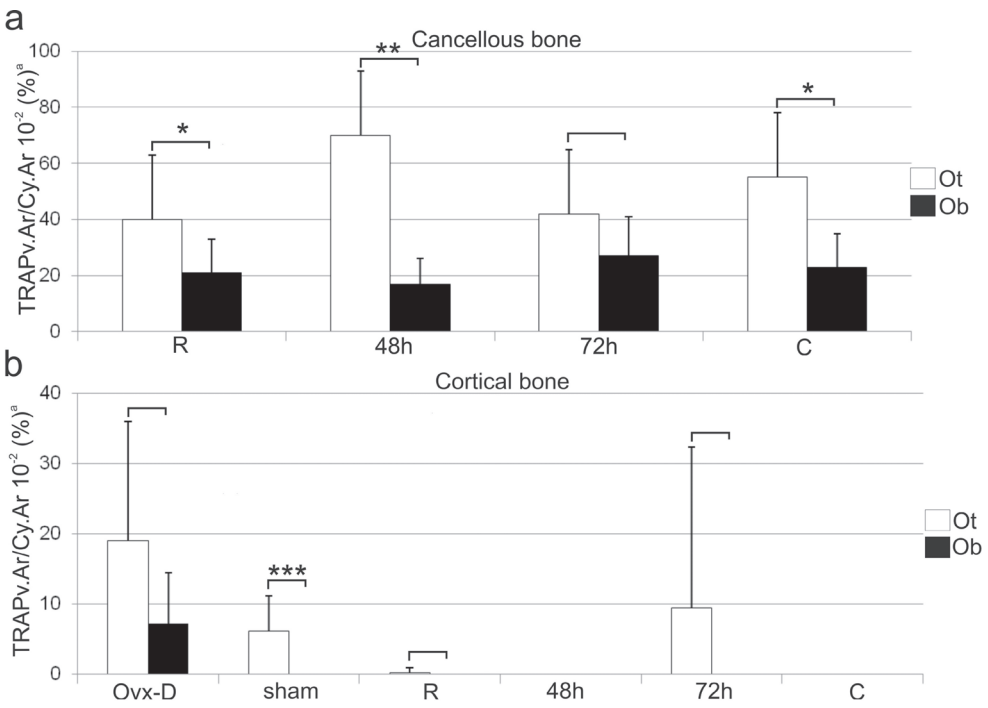
Online Resource 1 Animal models **a** Light microscopic images display a large difference in trabecular bone volume (BV/TV) between Ovx-D and sham. **b** BV/TV in femoral head and bone mineral density (BMD) in femoral neck and vertebrae were decreased in Ovx-D vs. sham (Student's t-test, n=7/7). **c** The animals with fulminant rickets demonstrated enlarged physis/metaphysis and lack of well-defined EMB, while a healing zone (He.Z) had developed

at 48h healing. After healing for 72h the animals showed almost a normal structure at the EMB but still with a small He.Z compared to controls. [a]The results are presented with mean and SD, [b]The results are means of the right and left limb, *** $p<0.001$

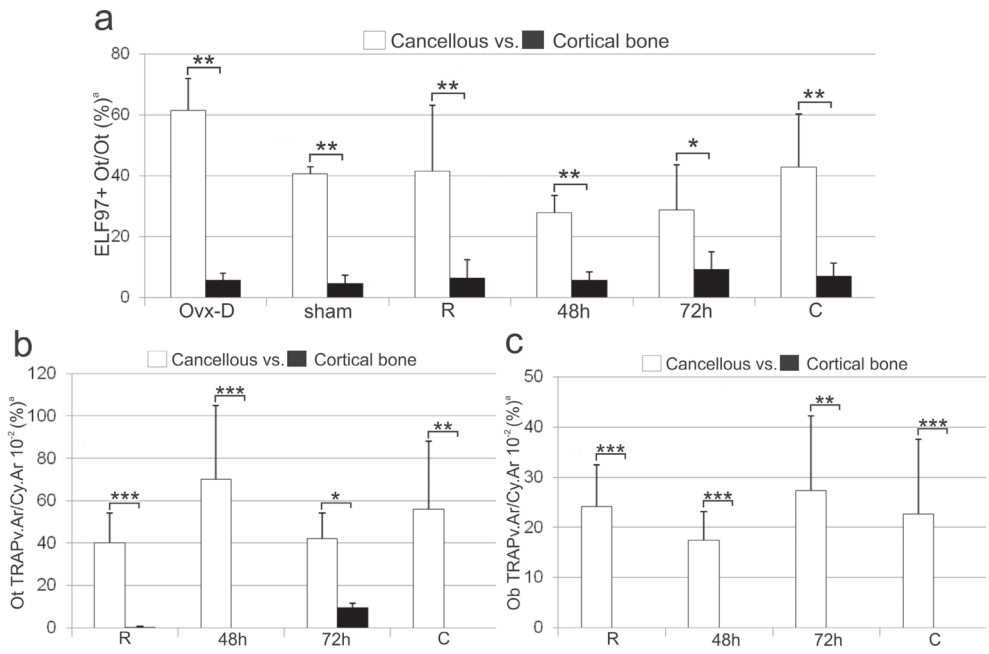


Online Resource 2 TRAP enzyme activity and monomeric TRAP (mTRAP) immunolabeling in osteocytes (Ot) in cortical bone from femur diaphysis. **a** HES stained sections of cortical bone in sham and **e** Ovx-D show the tissue architecture. The black outlines demonstrate

corresponding areas to the immunofluorescence images. **b-d** ELF97+ Ot (yellow-green, arrows), mTRAP+ Ot (red) and ELF97mTRAP+ Ot (arrowheads) in sham and **f-h** Ovx-D. **i** Inhibition of TRAP enzyme activity with molybdate demonstrated low background fluorescence for ELF97. **j** Unspecific rabbit IgG served as negative control for mTRAP with low background fluorescence. Scalebars 10µm



Online Resource 3 Histomorphometric semi-quantitative analyses of TRAPv.Ar/Cy.Ar in osteocytes (Ot) and osteoblasts (Ob). **a** TRAPv.Ar/Cy.Ar Ot vs. Ob in cancellous bone in experimental rickets (n=7/7/6/7). **b** TRAPv.Ar/Cy.Ar Ot vs. Ob in cortical bone in all groups (n=7/7/7/7/5/6) (b). The statistical analyses are performed with Mann-Whitney test. [a]The results are presented with mean and SD, ** $p<0.01$, *** $p<0.001$



Online Resource 4 TRAP expression increased in osteocytes (Ot) and osteoblasts (Ob) in cancellous vs. cortical bone. **a** TRAP enzyme activity in all groups displayed by ELF97+Ot/Ot. **b** TRAPv.Ar/Cy.Ar in Ot in experimental rickets. **c** TRAPv.Ar/Cy.Ar in Ob in experimental rickets. The statistical analyses are performed with Mann-Whitney test. [a]The results are presented with mean and SD, * $p<0.05$, ** $p<0.01$, *** $p<0.001$