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„The Role of Osteoclasts in Osteoarthritis: Tissue-
Dependent Phenotypes and Inhibition“

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Heiko Müller

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Betreut von: ao. Univ.-Prof. Mag. Dr. Ernst Müllner

Preface

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1. Abbreviations

ACLT	anterior cruciate ligament transection
ANOVA	analysis of variance
C2M	MMP-derived type II collagen neoepitope
Cat K	cathepsin K
CTR	calcitonin receptor
CTX-I	C-terminal telopeptide of type I collagen
CTX-II	C-terminal telopeptide of type II collagen
DC-STAMP	dendritic cell-specific transmembrane protein
DMM	destabilization of the medial meniscus
DMSO	dimethyl sulfoxide
E64	L-trans-epoxysuccinylleucylamido(4-guanido)butane
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GM6001	N-[2(R)-2-(hydroxamido carbonylmethyl)-4-methylpentanoyl]-L-tryptophane methylamide
hCT	human calcitonin
hOC	human osteoclast
HSC	hematopoietic stem cell
M-CSF	macrophage-colony stimulating factor
MMP	matrix metalloproteinase
MOPS	3-(N-morpholino)propanesulfonic acid
OD	optical density
PBS	phosphate buffered saline
PIIBNP	type II collagen N-propeptide
PTH	parathyroid hormone
RANK	receptor activator of nuclear factor κ B
RANKL	receptor activator of nuclear factor κ B ligand
sCT	salmon calcitonin
SDS	sodium dodecyl sulfate
SEM	standard error of mean
TBST	Tris-buffered saline + Tween 20
TCIRG1	T-cell immune regulator 1 / vacuolar H^+ ATPase V0 subunit a isoform 3
TRAcP	tartrate-resistant acid phosphatase
TRITC	tetramethyl rhodamine iso-thiocyanate
V-ATPase	vacuolar H^+ ATPase

2. Introduction

2.1 The tissues of the joint

In order to understand osteoarthritis in its full context, a basic understanding of the anatomy of joints is necessary. The joint comprises the components present at the location where two bones are connected. Its parts serve to connect, stabilize and protect the two bones and most importantly allow a friction-free movement of both. As the knee joint is often affected by osteoarthritis, I will focus on its description in the following.

The knee is the joint connecting the ends of femur (thigh bone) and tibia (shin bone). The condyles (rounded ends of the bone) of both bones are covered with a layer of articular cartilage. The shock-absorbing, cartilaginous lateral and medial menisci prevent the direct contact of femur and tibia. The space between the two bones is surrounded by the synovium and is filled with synovial fluid, a viscous substance secreted by the cells of the synovium. Apart from femur and tibia, two other bones are found in the knee, the patella (kneecap) and the fibula (more slender long bone next to the tibia). All bones in the knee are connected by ligaments which stabilize the structure. Tendons connect the leg muscles to the knee bones (Callaghan, 2003).

2.1.1 Articular cartilage

The articular cartilage covers the bones in the area of the joint. With its elastic properties, it reduces friction between the bones. The key to its elasticity lies in its structure, a meshwork of collagen fibrils and proteoglycans which retain a large amount of water. Tissue fluid can account for as much as 80 % of the total weight (Mow et al., 1992). The most abundant macromolecule which constitutes about 60 % of the dry weight is collagen, mostly of type II (types I, IV, V, VI, IX and XI are present in smaller amounts). The proteoglycans are mainly represented by aggrecan (Sophia Fox et al., 2009).

The articular cartilage is divided into three zones, the superficial zone, the middle zone and the deep zone (Figure 1). All of them are populated by cells called chondrocytes. They are responsible for the maintenance of cartilage and, therefore, secrete both proteases (matrix metalloproteinases and cathepsins) and matrix components. As there are no blood vessels and nerves present in cartilage, these cells rely on nutrients dissolved in the interstitial fluid (Sophia Fox et al., 2009).

2.1.2 Subchondral bone

Distinguished by the tidemark, the articular cartilage merges with the calcified cartilage, which in turn is connected to the subchondral plate (Havelka et al., 1984) (Figure 1). The calcified cartilage and the subchondral plate are commonly summarized as subchondral bone. However, despite this umbrella term, both tissues vary in their composition. About 65 % of the dry weight of calcified cartilage is represented by hydroxyapatite and about 20 % were identified as collagen type II. The subchondral plate, in contrast, does not contain collagen type II, but collagen type I (14 % of the dry weight); the hydroxyapatite amounts to 86 % (Zhang et al., 2012).

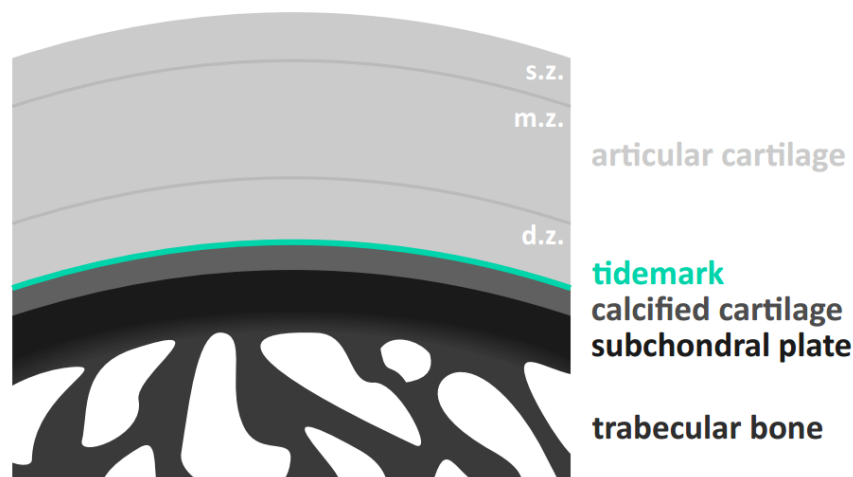


Figure 1. The structural zones of articular cartilage and the subchondral bone compartment. The calcified cartilage and the subchondral plate are commonly summarized as subchondral bone. Note, however, that no clear structural demarcation between subchondral plate and trabecular bone exists. s.z.: superficial zone, m.z.: middle zone, d.z.: deep zone.

Calcified cartilage was found to be resorbed by multinucleated osteoclast-like cells termed chondroclasts (Savostin-Asling & Asling, 1975). This allows blood vessels and nerves to invade from the underlying subchondral plate (Lane et al., 1977; Madry et al., 2010).

The subchondral plate contains several cell types. Osteocytes are found within the bone matrix, whereas the bone surface is populated with osteoclasts, osteoblasts and bone lining cells. These cells maintain the bone structure in a collaborative way, as I will explain in more detail later.

2.1.3 Trabecular bone

Below the subchondral plate, a less dense, porous type of bone is found (Figure 1). It is characterized by trabeculae and therefore usually called trabecular bone. Its structural characteristics allow for shock absorption in bones which are subject to mechanical stress (e.g. the condyles of femur and tibia). The trabeculae consist of plates connected by rods and are aligned along the direction of strains. They are continuously remodeled by osteoclasts and osteoblasts to adapt to altered stress levels and directionality (Chappard et al., 2008). Further, the trabeculae enclose the bone marrow including the hematopoietic stem cells (Kini & Nandeesh, 2012). Blood vessels provide the nutritional supply for the tissue. Nerves are also present in the bone marrow (Reimann & Christensen, 1977).

2.1.4 Cortical bone

Cortical bone is not actually part of the joint, but makes up the outer part of the shaft of long bones. Its high density and hardness enables it to resist the forces of body weight and prevents the bone marrow from injury (Seeman, 2008). Within the cortical bone, small canals are found, which harbor nerves and blood vessels. Similar to the trabecular bone, it is mostly made up of collagen type I and hydroxyapatite and undergoes continuous remodeling by the cells of the bone microenvironment (Buckwalter & Cooper, 1987). However, cortical bone is remodeled much slower than trabecular bone due to its smaller surface to volume ratio (Kini & Nandeesh, 2012).

2.1.5 Markers of joint tissue degradation

Osteoarthritis is marked by an increased destruction of joint components, especially the articular cartilage layer. Aberrant enzymatic breakdown of cartilage can, for example, be measured based on degradation products of collagen type II. Several classes of enzymes can cleave the molecule at different sites, which results in specific fragments. Cleavage in the C-terminal region, for example, yields the C-terminal telopeptide of type II collagen (CTX-II). It is generated by several matrix metalloproteinases (MMPs) as well as cathepsin B (Charni-Ben Tabassi et al., 2008). Another marker of cartilage degradation is the MMP-derived type II collagen neoepitope (C2M) (Bay-Jensen et al., 2011).

Enzymatic degradation of bone, on the other hand, can be measured by collagen type I fragments. The most prominent marker is the C-terminal telopeptide of type I collagen (CTX-I) (Bonde et al., 1997). It is presumably generated by cathepsins (Garnero et al., 2003). For *in vitro* studies of bone resorption, it is also feasible to determine extracellular calcium levels as a measure of bone resorption (Blair et al., 1986).

General collagen breakdown can be measured by hydroxyproline, a modified amino acid which is almost exclusively present in collagen and elastin (Jackson & Cleary, 1967).

2.2 Bone remodeling

After the development of the skeleton (i.e. the modeling) during the early stages of life, bones are not static. Instead, they undergo continuous remodeling to repair damage and adapt to biomechanical forces. This takes place in a spatially restricted association of osteoclasts, osteoblasts and osteocytes termed the basic multicellular unit (Frost, 1969). These cells work in coordination to substitute existing bone with new bone without a net loss or gain. The mechanosensitive osteocytes, which reside in the bone matrix, were found to report microdamage (Hazenbergh et al., 2006). In response to that, osteoclasts start to degrade the organic and inorganic components of the bone, leaving a pit in the bone surface. These pits are then filled up with bone matrix by cells called osteoblasts (Raisz, 1999).

Under physiological circumstances, the remodeling of bone in adults is a well balanced process of bone resorption and bone formation. Given a steady mechanical load, bone is, thus, neither gained nor lost. A coupling of both processes ensures that one cannot occur without the other. In particular, osteoclasts do not resorb bone without cytokines secreted by osteoblasts and they, in turn, do not form bone in the absence of osteoclasts (Matsuo & Irie, 2008). The latter is presumably caused by factors derived from the osteoclast directly as well as from the degradation of the bone matrix (Henriksen et al., 2013).

An uncoupling of osteoclasts and osteoblasts is observed in several pathological conditions. A net loss of bone is a hallmark of osteoporosis and osteoarthritis. Osteopetrosis, on the other hand, is characterized by impaired bone resorption due to mutational defects in osteoclast-relevant genes.

2.3 The osteoclast

The degradation of such solid tissue as bone or dentine requires a highly specialized type of cell. The osteoclast with its unique characteristics is the only cell capable of doing that. It is, therefore, essential for events such as bone growth, tooth eruption, fracture healing as well as the maintenance of blood calcium levels. Its absence or altered activity, on the other hand, can lead to a variety of bone diseases. As osteoarthritis is a disease of the elderly, I will focus my description on the osteoclast and its role in bone remodeling.

2.3.1 Osteoclastogenesis

Osteoclasts are derived from hematopoietic stem cells (HSC). The HSC gives rise to the myeloid stem cell, which can form the granulocyte-macrophage colony forming unit. It enters the circulation as a monocyte (Yavropoulou & Yovos, 2008). Following that, a number of environmental factors determine whether osteoclasts are formed. In the bone microenvironment, several cell types, such as osteoblasts, osteocytes and T-cells, release the macrophage-colony stimulating factor (M-CSF) as a reaction to high levels of parathyroid hormone (PTH), inflammatory molecules or possibly mechanical stress (Weir et al., 1996; Tu et al., 2015; Grcević et al., 2001). M-CSF promotes the proliferation of mononuclear osteoclast precursors and prevents apoptosis by binding to the colony stimulating factor 1 receptor. In the osteoclast, M-CSF triggers the expression of receptor activator of nuclear factor κ B transcription factor (RANK) (Kwon et al., 2005). The corresponding ligand (RANKL) is produced by osteocytes and osteoblasts, the latter of which present it as a membrane-bound form (Nakashima et al., 2011; Lacey et al., 1998). Binding of RANKL to RANK on osteoclasts triggers several transcriptional pathways leading to the expression of cathepsin K (Cat K), tartrate-resistant acid phosphatase (TRAcP), calcitonin receptor (CTR), the α 3 isoform of the vacuolar ATPase V0 domain (TCIRG1) and dendritic cell-specific transmembrane protein (DC-STAMP) (Yavropoulou & Yovos, 2008). Facilitated by proteins, such as DC-STAMP, the mononuclear osteoclast can now fuse with other osteoclasts. This happens by “cellocytosing”, where the master-fusing cell endocytoses the other to form a large, multinucleated, mature osteoclast, which is now capable of effectively resorbing bone (Yagi et al., 2005).

In vitro, this differentiation process can be reproduced in the absence of osteoblasts or T-cells when monocytes are cultured in the presence of recombinant M-CSF and RANKL (Sørensen et al., 2006).

2.3.2 Bone resorption

In order to resorb bone, the osteoclast tightly attaches to the bone surface and polarizes to form several functional membrane domains (Figure 2). The space between the cell and the bone matrix is called the resorption lacuna; this is where the degradation of bone takes place. The part of the cell membrane which covers the lacuna is very irregularly shaped and thus termed the ruffled border. It is the interface through which the components needed for the resorption of bone are released and the degradation products are taken up. The ruffled border is confined by the sealing zone. This is the part of the membrane which attaches the cell to the bone and ensures the tight sealing of the resorption lacuna necessary for the effective resorption of bone. The cell membrane facing away from the bone comprises two domains, namely the basolateral membrane and the functional secretory domain. At the functional secretory domain, transcytosed degradation products exit the cell (Baron, 1989; Väänänen et al., 2000; Domon et al., 2002). In all of these domains essential molecular processes are taking place during the resorption cycle.

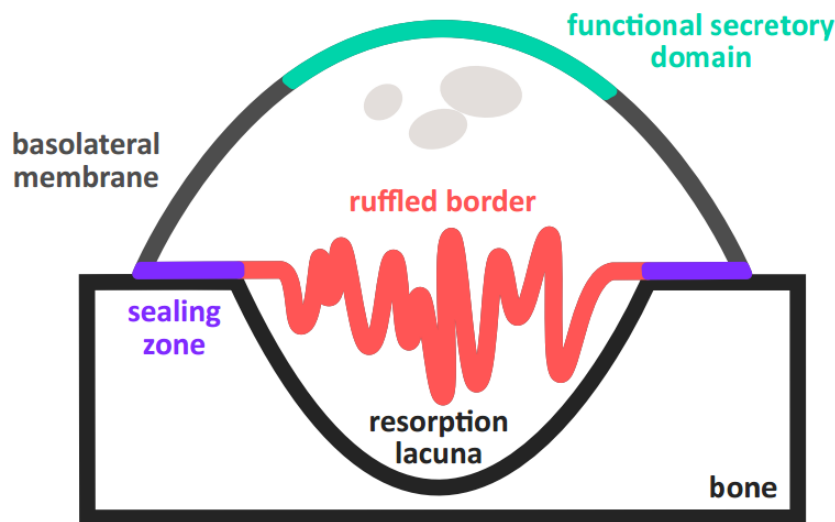


Figure 2. The functional domains of a resorbing osteoclast.

Once the osteoclast recognizes the bone surface where bone is to be resorbed, it starts to form podosomes, which attach the cell to the extracellular matrix. They contain transmembrane receptors such as integrin and CD44, which, extracellularly, bind to a variety of matrix proteins, such as collagen. Intracellularly, they are linked to a network of actin filaments via adaptor proteins such as vinculin, paxilin and talin (Schachtner et al., 2013). After a while, the podosomes assemble around the border of the cell to form a ring structure, the sealing zone. It can easily be visualized, for example, by staining for F-actin and serves as an indicator for resorption-potent osteoclasts (Väänänen & Horton, 1995). Fusion of lysosomal vesicles with the membrane encircled by the sealing zone leads to the protrusions of the ruffled border. These vesicles acidify the resorption lacuna by direct release of acid as well as the insertion of the vacuolar H^+ ATPase (V-ATPase) and the chloride channel type 7 into the membrane (Blair et al., 1989; Schlesinger et al., 1997). Together, they further decrease the pH in the resorption lacuna through the release of hydrochloric acid. The low pH is sufficient to dissolve the hydroxyapatite of the bone, but not the organic matrix. A mixture of proteases is, therefore, delivered to the resorption lacuna by exocytosis. Among them are MMPs, TRAcP 5b and the papain-like cystein proteinase Cat K (Figure 3) (Baron et al., 1988; Stenbeck, 2002). Whereas Cat K plays a central role in the degradation of collagen type I, with mutations of its gene leading to pycnodysostosis (Zaidi et al., 2001; Gelb et al., 1996), the role of MMPs is less clear (Henriksen et al., 2006). Also unknown is how TRAcP 5b participates, but its specificity to osteoclasts facilitates its use as a marker for the number of mature osteoclasts (Alatalo et al., 2000).

The demand for hydrochloric acid for the dissolution of the inorganic matrix of bone is satisfied by the generation of carbonic acid by the carbonic anhydrase II (Sly et al., 1983). The Cl^-/HCO_3^- exchanger provides the chloride ions and clears the cell from excess bicarbonate (Wu et al., 2008).

The products of bone resorption are taken up into the cell by endocytosis and are released at the basal side of the cell at the functional secretory domain (Salo et al., 1997). During the process of transcytosis, the resorption fragments might undergo further degradation (Vääräniemi et al., 2004).

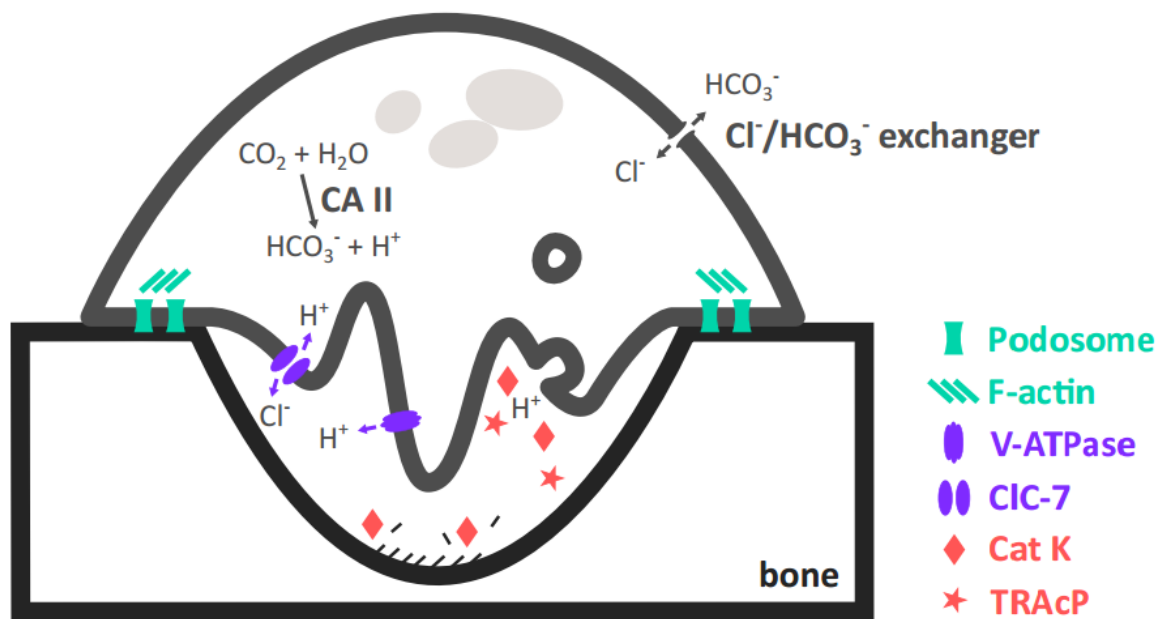


Figure 3. The molecular key players of osteoclastic bone resorption. CA II: carbonic anhydrase II, Cat K: cathepsin K, CIC-7: chloride channel type 7, TRAcP: tartrate-resistant acid phosphatase, V-ATPase: vacuolar H⁺ ATPase.

2.3.3 The osteoclast in bone pathologies

Osteoclasts carry out a central role in the metabolism of bone. Consequently, dysfunctional or hyperfunctional osteoclasts can lead to a variety of bone diseases. Excessive bone resorption leading to a decreased bone mass is observed in Paget's disease and osteoporosis. In contrast, osteopetrosis is characterized by impaired bone resorption and elevated bone mass, due to mutational defects in osteoclast-relevant genes (Raisz, 1999). Two phenotypes of the disease exist, the osteoclast-rich and the osteoclast-poor subtype. Mutations directly abrogating osteoclastic resorption lead to an increase in osteoclast numbers, whereas mutations affecting RANK or RANKL prevent the formation of osteoclasts (Balemans et al., 2005). The coupling of osteoclasts and osteoblasts is demonstrated by the fact that bone formation is increased in the osteoclast-rich phenotype, while it remains at baseline levels in the osteoclast-poor subtype (Thudium et al., 2014). Why bone formation is not further reduced in the osteoclast-poor subtype remains to be investigated.

2.4 Osteoarthritis

Osteoarthritis is the most prevalent arthritic condition (Abramson et al., 2006) and is marked by a destruction of cartilage, remodeling of the subchondral compartment and synovitis in joints. It is uncommon in people under the age of 40, but quite common in people over the age of 65 (Loeser, 2010). Due to its irreversibility, it affects most people over the age of 70 (Petersson & Jacobsson, 2002). Apart from increasing age, obesity (Felson et al., 1992), joint injury (Gelber et al., 2000) and genetic dispositions (Holderbaum et al., 1999) represent additional risk factors. Patients suffering from the disease experience a swelling and limited mobility of the affected joint, which is accompanied by pain. The joints which are mainly affected are the knees and the hip, but others are not excluded. Up to now, the lack of pharmaceutical ways of intervention restricts therapy options to palliative care or surgical

replacement of the joint in more severe cases. An aging and increasingly obese society further fuels efforts in finding a disease-modifying drug.

2.4.1 Anatomic and molecular pathology

The histological changes in osteoarthritis are not merely caused by mechanical abrasion. Instead, various processes on the molecular level, partly in an attempt to compensate for altered biomechanics, contribute significantly to the pathology. They take place in several tissues of the joint in a potentially collaborative manner, thus making osteoarthritis a disease of the whole joint. The most prominent symptom is the loss of articular cartilage which results in a narrowing of the joint space. This is thought to be caused by hypertrophic chondrocytes in the articular cartilage; they produce abnormal amounts of collagen type X and proteases (Von der Mark et al., 1992; Van der Kraan & Van den Berg, 2012) and promote the calcification of the articular cartilage (Hough, 2001).

Moreover, an inflammation of the synovium is found in at least 50 % of osteoarthritis patients and evidence points at an involvement of T-cells (Sakkas & Platsoucas, 2007).

The remodeling of the subchondral compartment is characterized by subchondral bone sclerosis, osteophyte formation, bone marrow lesions, trabecular thinning, vascularization of the subchondral bone and calcified cartilage as well as the presence of nerve endings in osteophytes (Karsdal et al., 2015) (Figure 4) This extensive remodeling of bone has been discussed as a possible factor driving cartilage pathology (Karsdal et al., 2008). Evidence for that was already found in 1993, when Dieppe et al. observed that bone abnormalities in patients with osteoarthritis could predict the progression of the disease (Dieppe et al., 1993). In more recent years, a number of studies of animal models strengthened this hypothesis. In a rat model of osteoarthritis, inhibition of bone resorption was found to reduce cartilage pathology significantly (Hayami et al., 2004). In contrast, increased bone loss due to induction of osteoporosis in rabbits before inducing osteoarthritis led to more severe cartilage damage (Calvo et al., 2007). These findings indicate a critical role for the remodeling of subchondral bone in the pathogenesis of osteoarthritis. Osteoclasts, as the only bone resorbing cells, might, thus, play a central role in this disease.

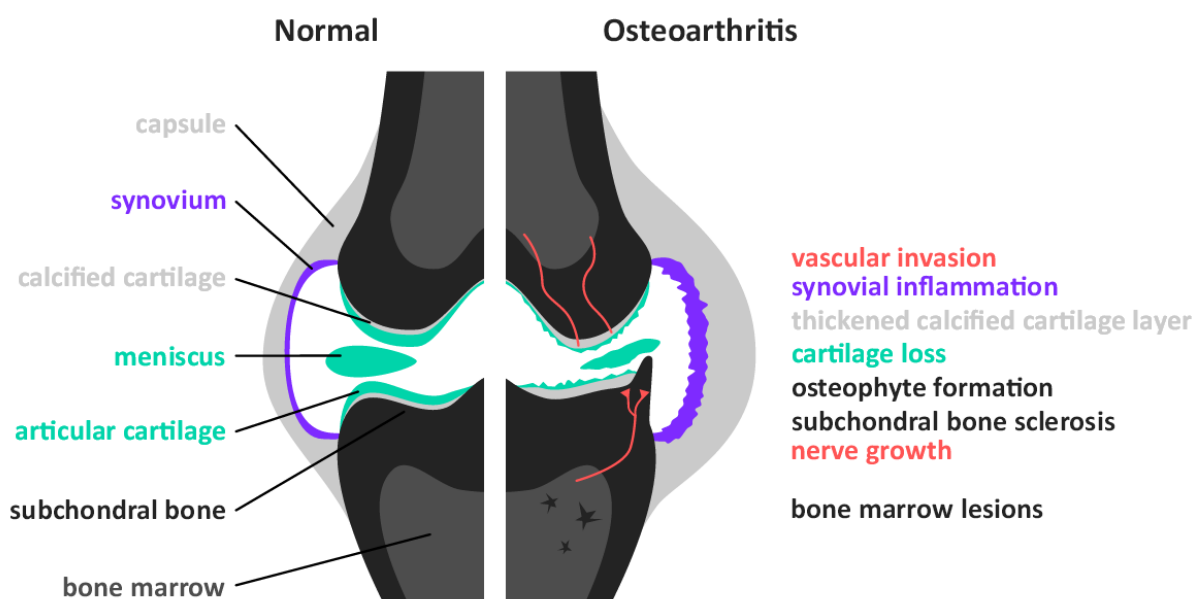


Figure 4. Pathological features of osteoarthritis.

2.4.2 The role of osteoclasts in osteoarthritis

Multinucleated clastic cells are present throughout the subchondral bone compartment. Osteoclasts contribute to the maintenance of the bone structure. The closely related chondroclasts reside on calcified cartilage and presumably contribute to endochondral ossification (Wlodarski et al., 2014). In arthritic conditions, chondroclasts were found to resorb the calcified cartilage layer possibly facilitating vascular and neuronal infiltration (Knowles et al., 2012). Moreover, alterations in the subchondral bone are a common hallmark of osteoarthritis and increased levels of bone resorption markers are predictive of osteoarthritis progression (Hunter & Spector, 2003). These findings suggest a role for osteoclasts in osteoarthritis. Indeed, inhibiting them was found to reduce pathological parameters *in vivo* (Hayami et al., 2004; Hayami et al., 2012). Counterintuitively, osteopetrosis, a disease marked by a lack of bone resorption, has been associated with premature osteoarthritis (Casden et al., 1989).

The exact way in which osteoclasts participate to the pathogenesis of osteoarthritis remains to be investigated. This includes the study of their behavior on the subchondral tissues and articular cartilage. Apart from its bone resorptive function, direct signaling to other cells, such as osteoblasts, osteocytes and chondrocytes might play a role as well. Especially the coupling to osteoblasts needs to be considered. Both *in vitro* experiments with pure osteoclast cultures as well as *in vivo* experiments for the study of osteoclasts in their physiological environment may, therefore, provide valuable insights in the role of osteoclasts in osteoarthritis.

2.5 Calcitonin

Calcium is an important ion for all vertebrates, as it plays an essential role in many biological processes. The extracellular concentration of calcium is, thus, tightly regulated by hormones (Pang, 2014). PTH is released from the parathyroid gland upon hypocalcemia. It stimulates osteoclasts to resorb bone and, thus, calcium release (Raisz, 1999). Calcium induces the release of calcitonin from the parafollicular cells in the thyroid gland which directly inhibits osteoclastic bone resorption (Care et al., 1968; Hirsch et al., 1964; Friedman & Raisz, 1965). Therefore, in past decades, it was proposed as a counter-regulator to PTH in calcium homeostasis. However, in physiological doses, calcitonin only exerts a very weak and transient effect on serum calcium levels and researchers still speculate about its physiological role (Davey & Findlay, 2013; Hirsch et al., 2001).

Calcitonin in its mature form is a 32 amino acid peptide, which arises from one gene in a family of five calcitonin genes (CALC-I to CALC-V). This gene family also encodes other peptides, such as amylin, calcitonin gene-related peptide and adrenomedullin. Except for adrenomedullin, these peptides have all been shown to inhibit osteoclastic bone resorption (Lerner, 2006).

Calcitonin binds to the CTR, a G-protein coupled receptor first cloned in 1991 (Lin et al., 1991). The CTR is not expressed in bone marrow macrophages, but becomes upregulated in osteoclast differentiation induced by RANKL (Granhölm et al., 2008). It signals via pathways such as the cyclic adenosine monophosphate / protein kinase A or protein kinase C pathway and the mitogen-activated protein kinase pathway (Suzuki et al., 1996; Samura et al., 2000; Zhang et al., 2002). In osteoclasts, it particularly impairs cell motility. Through interaction with receptor modifying proteins, the CTR can convert into an amylin receptor (Christopoulos et al., 1999).

Not long after its discovery, calcitonin was found to inhibit osteoclastic bone resorption *in vitro* and *in vivo* (Friedman & Raisz, 1965; Johnston & Deiss, 1966). The ability to induce the storage of calcium in bones together with phosphate indicated an additional anabolic effect of calcitonin (Talmage et al., 1981). These discoveries suggested its use as a drug for diseases marked by a progressive loss of bone. The first clinical application for human calcitonin (hCT) was for the treatment of Paget's disease where it reduced serum calcium and phosphatase levels (Woodhouse et al., 1971). Soon, however, its teleost homolog took over the stage.

2.5.1 Salmon calcitonin and the analogous KBP-056

Soon after the discovery of calcitonin, comparative endocrinology led to the discovery of salmon calcitonin (sCT) and revealed its significantly increased potency over human and porcine calcitonin in inducing hypocalcemia *in vivo* (Sturtridge & Kumar, 1968). As a result, sCT was approved for the treatment of Paget's disease by the FDA in 1975 (Fatourechhi & Heath, 1987). Later, beneficial effects of sCT in the treatment of postmenopausal osteoporosis have been found (Mazzuoli et al., 1986) and the FDA approved it for the treatment of osteoporosis in 1985 (Fatourechhi & Heath, 1987).

Apart from positive effects on bone metabolism, protective and anabolic effects of calcitonin on cartilage have been described. First evidence for a chondroprotective effect was provided by a reduction of urinary hydroxyproline levels (Klein & Talmage, 1968). This might be due to the presence of the CTR on articular chondrocytes and the inhibition of collagen type II degradation through decreased MMP expression (Sondergaard et al., 2006b; Sondergaard et al., 2007). Moreover, a possible anabolic effect of calcitonin on cartilage formation and synthesis was described (Baxter et al., 1968). As osteoarthritis is characterized by a loss of bone and cartilage, calcitonin seems like a well suited treatment option. However, a recently developed oral formulation of sCT failed to show a clinical benefit in the treatment of osteoarthritis, possibly due to the formulation or a limited efficacy of sCT in humans (Karsdal et al., 2015).

About 50% of patients produce antibodies against sCT (Singer et al., 1972) which might be relevant in the development of resistance (Grauer et al., 1995). A pharmacologically limited efficacy encouraged the search for more potent derivatives of sCT. Of particular interest in my thesis is KBP-056, a salmon calcitonin analog developed at Nordic Bioscience. Since other KBPs do not lie within the scope of this thesis, I will simply refer to it as 'KBP' hereafter.

The superiority of sCT over hCT is not fully resolved on a molecular level yet. It was, however, shown that sCT differs from mammalian calcitonins in its ability to bind not only to the CTR, but also to the amylin receptors with high affinity (Christopoulos et al., 1999). Both receptors are present in mature osteoclasts and both ligands have been found to inhibit osteoclastic bone resorption (Lerner, 2006). Moreover, recent evidence suggests that receptors activated by sCT remain active for a longer time even if they are internalized (Andreassen et al., 2014).

Taken together, finding a more efficient sCT analog while preserving the beneficial effects to bone and cartilage might eventually lead to the first disease-modifying drug for osteoarthritis. To determine whether KBP could be the molecule searched for, a series of tests *in vitro* and *in vivo* is necessary to confirm its superiority over sCT.

2.6 The aim of this project

Osteoarthritis is a rather complex disease with biomechanical as well as molecular factors involved in its development and progression. It affects several tissues of the joint and is characterized by the participation of several cell types. However, the temporal and causal order of events is largely unknown. The lack of a disease-modifying drug for osteoarthritis as well as a heterogeneous patient population ask for a more differentiated view on this disease of the whole joint and the cells and tissues involved in it. In my master thesis, I, thus, focused on the osteoclast and its role in osteoarthritis. In particular, the aim was to elucidate the osteoclast's response to different matrices and pharmaceutical interventions *in vitro* as well as the characterization of an osteoarthritis mouse model lacking osteoclastic bone resorption. A brief summary of the rationale of my project, divided into three parts, is given in the following.

2.6.1 Evaluation of osteoclast phenotypes on osteoarthritis-relevant joint tissues

Osteoarthritis is marked by alterations in and destruction of several compartments of the joint. On bone, osteoclasts carry out an important physiological role. Whether they act on other tissues as well, possibly contributing to the pathology is, however, unclear. Understanding the influence of the osteoclast, as a potential target of future disease-modifying drugs, requires the determination of the tissues it acts on. In order to shed light on this question, I intended to study the influences of bovine cortical bone, calcified cartilage and articular cartilage on osteoclasts in terms of their viability, resorptive activity and expression of resorption-relevant proteins.

2.6.2 Effects of KBP on osteoclastic bone resorption

Calcitonins are potent inhibitors of osteoclastic bone resorption. A synthetic version of sCT has been used in the clinic for several decades now to treat bone diseases marked by a net loss of bone, such as osteoporosis and Paget's disease. More recently, there have been indications that it might be suitable for the treatment of osteoarthritis, as well. In the second part of my project, I wanted to assess the efficacy of the sCT analog KBP (Nordic Bioscience, Herlev, Denmark) in inhibiting osteoclastic bone resorption *in vitro*. Ideally, I then wanted to investigate the effect of the peptide on osteoclasts cultured on different osteoarthritis-relevant tissues.

2.6.3 Characterization of a resorption-impaired mouse model of osteoarthritis

In recent years, evidence for a disease-promoting role of the bone compartment in osteoarthritis has accumulated, suggesting a critical role for the osteoclast in the progression of the disease. In order to shed light on its involvement in the changes of subchondral bone and articular cartilage seen in osteoarthritis, we wanted to study an artificial *in vivo* model of osteoarthritis lacking osteoclastic bone resorption. To this end, the hematopoietic system of mice was substituted with stem cells isolated from osteopetrotic mouse models, defined by either dysfunctional (i.e. non-resorbing) osteoclasts or lack of osteoclastogenesis. Osteoarthritis was induced by destabilization of the medial meniscus (DMM) in one knee. The model should then be characterized by measuring serum markers of bone and cartilage turnover as well as histological inspection of cartilage destruction and ectopic presence of nerves and blood vessels.

3. Materials and methods

A comprehensive list of materials and buffer / media recipes can be found in the appendix.

3.1 Preparation and culture of human osteoclasts

3.1.1 Isolation of CD14-positive monocytes from human peripheral blood

For all *in vitro* experiments, I used freshly prepared human osteoclasts (hOCs). They were generated through differentiation of monocytes obtained from human peripheral blood. Therefore, a super-buffycoat of approximately 150 ml containing blood from four donors of the same blood group was received from Rigshospitalet (Copenhagen, Denmark). The blood was diluted with phosphate buffered saline (PBS) to 200 ml. In eight 50 ml conical tubes, 25 ml of the diluted blood were carefully layered on top of 25 ml of ficoll. The tubes were centrifuged at 400 g for 20 min (brake deactivated). The white mononuclear cell layer was transferred to new tubes, diluted with 45 ml of PBS and centrifuged at 400 g for 12 min. After removal of the supernatant, the cells were resuspended in 45 ml PBS and centrifuged again at 400 g for 12 min. The supernatant was removed and all cells were collected in 30 ml of PBS containing 2 % fetal bovine serum (FBS).

The cell suspension was distributed equally among six conical tubes containing 125 μ l CD14 beads previously washed in PBS three times. The cells were then incubated with the beads on an end-over-end shaker for 20 min at 4 °C. Using a magnetic particle concentrator, the cells were washed six times with PBS containing 2 % FBS. Afterwards, the cells were resuspended in 40 ml RANKL-negative hOC medium, counted and seeded in cell culture flasks at a density of 150,000 cells/cm².

3.1.2 Differentiation of monocytes into osteoclasts

To differentiate monocytes into osteoclasts, the cells have to be cultured in the presence of M-CSF and RANKL. To this end, freshly isolated monocytes were cultured in RANKL-negative hOC medium for three days (37 °C and 5 % CO₂). Thereafter, RANKL-negative medium was substituted for RANKL-positive hOC medium within which the cells were cultured for another 8 - 10 days. Every other day, the medium was exchanged for fresh medium.

3.1.3 Preparation of osteoclasts for experiments

In order to use the osteoclasts in further experiments, they were lifted by trypsinization. The cells were washed in cold PBS two times and 40 μ l/cm² trypsin were added. After incubation at 37 °C for 10 min, the cells were scraped off and the trypsinization stopped by addition of RANKL-negative hOC medium. In conical tubes, the cells were then centrifuged at 400 g for 5 min. The supernatant was removed and the cells were pooled in 7 – 10 ml of RANKL-positive hOC medium. To determine the amount of cells, they were counted using a Neubauer chamber and diluted to the required seeding density.

3.2 In vitro bone resorption experiments

3.2.1 Preparation of cortical and subchondral bone matrices

Sticks of cortical and subchondral bone with a diameter of 5 mm obtained from the femoral shaft and the femoral condyle of old cows, respectively were available from a previous project. They were stored in 70 % ethanol at 4 °C. To produce slices of 0.65 mm thickness, the sticks were sectioned using a minitome. The rough edges on the resulting slices were cut off with a scalpel and the slices were put in 70 % ethanol and were sonicated for 30 min. Afterwards, the slices were washed three times in 70 % ethanol and irradiated in a UV oven for 7 min. Until further use, the slices were stored in 70 % ethanol in a parafilm-sealed petri dish at 4 °C.

3.2.2 Preparation of articular cartilage matrix

Slices of bovine articular cartilage with a diameter of 5 mm were obtained by punch biopsy from the femoral condyle of old cows in a previous project. They were metabolically inactivated in liquid nitrogen and stored in 70 % ethanol at 4 °C. Before using the slices in my experiments, I repeated the metabolic inactivation. Thus, I washed the slices in PBS, transferred them to a conical tube, removed the PBS and put them into liquid nitrogen for 1 min. Afterwards, they were washed with 70 % ethanol and stored at 4 °C.

3.2.3 Seeding of cells on matrices

The bone or cartilage slices were washed in RANKL-negative hOC medium five times. 150 µl of medium were added to the wells of a 96-well plate. With forceps, the slices were then transferred to the 96-well plate and gently pushed to the bottom of the well.

For differentiation experiments, freshly isolated CD14-positive monocytes were diluted to 500,000 cells/ml for seeding them on matrix or 250,000 cells/ml for seeding them on plastic.

To study mature osteoclasts, osteoclasts trypsinized as described above were diluted to 150,000 – 225,000 cells/ml, depending on the experiment.

The medium was removed from the wells and 200 µl of cell suspension were added to the wells. Empty wells were filled with PBS to reduce evaporation. The cells were incubated at 37 °C, 5 % CO₂. The supernatant was collected for analysis every 2 - 3 days and replaced with fresh RANKL-positive hOC medium or the respective inhibitor diluted in medium.

3.2.4 Addition of inhibitors

After seeding the cells on matrices, they were usually allowed to settle for 24 h before inhibitors were added. The medium was then aspirated and 200 µl of the respective inhibitor diluted in RANKL-positive hOC medium were added.

KBP and sCT were diluted in deionized water to a concentration of 100 µM, diphyltin was diluted in dimethyl sulfoxide (DMSO) to a concentration of 10 mM, GM6001 was diluted in DMSO to a concentration of 20 mM and E64 was diluted in PBS to a concentration of 20 mM. Further dilution steps down to the working concentration were done using RANKL-positive hOC medium.

In KBP / calcitonin experiments, RANKL-positive hOC medium was used as a vehicle control, whereas in the study of osteoclast phenotypes on different matrices the vehicle control was represented by 1:2000 DMSO in RANKL-positive hOC medium.

For experiments spanning several days, supernatants were collected and replaced by fresh inhibitor dilutions every 2 – 3 days.

3.2.5 Cell viability assay

To obtain an estimate of the toxicity of the matrices to mature osteoclasts, an alamarBlue assay was performed at the end of experiments involving other matrices besides cortical bone or plastic. To this end, after collection of the supernatant, 200 µl of 10 % alamarBlue reagent in RANKL-positive hOC medium were added to each well with cells and to six cells without cells as a blank measurement. After incubation at 37 °C for 3 h, 180 µl of supernatant were transferred to a black 96-well plate and the fluorescence was measured (ex.: 540 nm, em.: 590 nm).

3.3 Examination of osteoclastic protein expression on different matrices

3.3.1 Protein isolation

To evaluate a shift in protein expression of osteoclasts dependent on the matrix they reside on, mature hOCs (45,000 cells/well) were cultured on cortical bone, subchondral bone and articular cartilage for 2, 5 or 7 days. Afterwards, the cells were washed on ice with cold PBS once and then lysed in 50 µl of RIPA buffer containing protease inhibitors 1:200. After 30 min of incubation, the cells were scraped off the matrix (cortical bone and articular cartilage) or washed off by pipetting (subchondral bone). The lysates of 20 replicates per condition were pooled and centrifuged at 12,000 rpm and 4 °C for 20 min. The supernatant was kept at -20 °C until further use.

3.3.2 Electrophoretic protein separation and blotting

Before the electrophoretic separation of protein lysates, the protein concentration was determined with the BioRad DC kit according to the manufacturer's instructions.

A volume of lysate containing 9 g of protein was mixed with 5 µl NuPage LDS sample buffer (4x), 2 µl NuPage Reducing Agent (10x) and deionized water to a total volume of 20 µl. The samples were heated to 70 °C for 10 min and then loaded on a NuPage Bis-Tris 4 – 12 % gel. 5 µl of Rainbow marker was run as a reference of protein size. The gel was run for about 45 min at 200 V and 125 mA in a NuPage MOPS SDS running buffer (1x) at room temperature. Hydrated in Tris-buffered saline with Tween 20 (TBST), the gel was transferred to an iBlot device and proteins were blotted on a membrane. The membrane was then stained in Ponceau red solution and photographed. It was then blocked over night in 5 % skim milk powder in TBST at 4 °C.

3.3.3 Immunological detection of resorption-relevant proteins

For the immunological detection of TCIRG1, p38, Cat K and MMP-9, the membrane was incubated in a 1:1000 dilution of the respective primary antibody in 5 % skim milk powder in TBST over night at 4 °C. The next day, the membrane was washed in TBST 3 times for 20 min. Next, the membrane was incubated with secondary antibody (TCIRG1: anti-mouse 1:10,000, p38: goat anti-rabbit 1:5000, Cat K / MMP-9: goat anti-rabbit: 1:10,000) in 5 % skim milk powder in TBST for 1 h at 4 °C. Subsequently, the membrane was washed 3 times in TBST for 20 min and was then ready for detection.

Between the detection of different target proteins the membrane was stripped and the blocking repeated. To this end, the membrane was incubated in stripping buffer containing

7 µl 2-mercaptoethanol per ml of buffer for 30 min. The membrane was then washed 3 times in TBST for 20 min and blocked in 5 % skim milk powder in TBST for at least 1 h.

For the chemical detection of antibody bound to the membrane, the membrane was incubated in a 1:1 mixture of Western Blot Detection Reagents for about 1 min. The liquid was removed and a film was put on the membrane for 2.5 min (TCIRG1), 1 min (p38), 34 min (Cat K) or 2 sec (MMP-9). Finally the film was developed.

3.4 Determination of marker levels in supernatants of in vitro resorption experiments

3.4.1 Calcium concentration

For the determination of extracellular calcium levels in bone resorption experiments as a measure of osteoclastic bone resorption activity, 100 µl of supernatant were sent to Nordic BRL (Rødovre, Denmark) for spectrophotometric analysis.

3.4.2 CTX-I concentration

The determination of the collagen type I degradation marker CTX-I in culture supernatants from resorption experiments was performed using the commercial Serum CrossLaps ELISA, lot no.: C0207A (Nordic Bioscience, Herlev, Denmark). The sample concentrations were determined from a standard curve ranging from 0 to 2.505 ng/ml with a four-parametric fit.

3.4.3 CTX-II concentration

Collagen type II degradation in osteoclast culture supernatants was determined by an ELISA targeting CTX-II. The sample concentrations were calculated from a standard curve ranging from 0 to 7.17 ng/ml with a four-parametric fit.

3.4.4 C2M concentration

C2M, a marker for metalloproteinase-mediated collagen type II degradation, was determined in supernatants from resorption experiments with the C2M ELISA, lot no.: FC1404A (Nordic Bioscience, Denmark). The sample concentrations were determined from a standard curve ranging from 0 to 2.435 ng/ml with a four-parametric fit.

3.4.5 TRAcP activity

As a measure of osteoclast numbers, TRAcP activity was determined. Thus, 20 µl of culture supernatant were transferred to a 96-well plate. If necessary, it was diluted in deionized water. After addition, of 80 µl TRAcP solution buffer, the solution was carefully mixed, sealed and, protected from light, incubated at 37 °C for 1 h. The reaction was stopped by addition of 100 µl 0.3 N sodium hydroxide and the absorbance was measured using a plate reader (405 nm / 650 nm).

If the OD value was above 2.5, the sample was measured again in a more diluted form. For better comparison, the OD value was then corrected for the dilution factor.

3.5 Cytochemistry

As soon as a resorption experiment ended and the supernatant of the last time point was collected, the cells were fixed with 4 % formaldehyde for 20 min. Afterwards, the cells were washed 2 - 3 times with PBS and stored at 4 °C until staining.

3.5.1 Resorption pits

To check cortical bone matrices incubated with osteoclasts for resorption pits, the slices were washed two times with deionized water and incubated with 150 µl hematoxylin for 7 min. The slice was then transferred to a paper towel and rubbed with a cotton swab leaving just the pits stained. After transferring the slice back to a microwell assay plate and covering it with 150 µl deionized water, the pits could be observed on the microscope.

3.5.2 Actin

Actin staining was performed to visualize the actin rings characteristic for resorption-potent osteoclasts. Therefore, the formaldehyde-fixed cells were permeabilized by incubating them in 0.1 % Triton X-100 for 30 min. Subsequently, tetramethyl rhodamine iso-thiocyanate (TRITC)-conjugated phalloidin was added to a final dilution of 1:1000 and the cells were incubated for 1 h in the dark at room temperature. After washing them 2 - 3 times in PBS, they were ready for microscopy.

3.5.3 TRAcP

To show cells which produce the osteoclast marker TRAcP, the Leukocyte Acid Phosphatase Kit (Sigma-Aldrich) was used. 50 µl Fast Garnet and 50 µl NaNO₂ were mixed and left for 7 min. The solution was then mixed with 4.5 ml deionized water, 50 µl naphtol, 200 µl acetate solution and 100 µl tartrate. 55 µl of this mixture were then added to the matrix and incubated for 8 - 9 minutes. The matrices were washed three times with tap water and stored in 150 µl PBS at 4 °C.

3.6 Generation of the resorption-impaired osteoarthritis mouse model

To study the role of the osteoclast in osteoarthritis *in vivo*, a mouse model combining osteoarthritis with either osteoclast-poor or osteoclast-rich osteopetrosis was generated before I started my thesis. In particular, the hematopoietic system of at least 3 months-old C57BL/6 mice was extinguished by irradiation. Subsequently, through injection into the tail vein, they were transplanted with 2 million fetal liver cells from 14.5 days-old mouse fetuses with one of the following genotypes obtained by breeding either RANK^{+/-} (obtained as described by Dougall et al., 1999) or TCIRG1^{+/-} (B6C3Fe a/a-Tcirg1^{oc}/J; stock number 000230) mice (Thudium et al., 2014):

- RANK^{-/-}
- RANK^{+/-} or wild type (control)
- TCIRG1^{-/-}
- TCIRG1^{+/-} or wild type (control)

Heterozygotes are haplosufficient and, thus, serve as controls. The transplantation efficiency was determined from the ratio of donor- and host-specific cell surface markers measured by fluorescence-activated cell sorting analysis of blood samples.

Six weeks after the transplantation, surgical DMM was performed in the right knee. After 15 weeks, the mice were euthanized and the knee joints were fixed in 4 % formaldehyde for 3 days. The knees were decalcified in 15 % ethylenediaminetetraacetic acid (EDTA) and 0.5 % formaldehyde (pH 7.4) for 1 week, followed by 15 % EDTA (pH 7.4) for 2 weeks. Finally, they were infiltrated with paraffin and stored at 4 °C until further use.

3.7 Preparation of mouse knees for histology

3.7.1 Embedding

Mouse knee specimens consisting of the joint and about half of the femur as well as tibia were fixed to the bottom of a metal cup with a small amount of liquid paraffin so that the joint was touching the bottom and the bones were pointing away from the bottom in a V-shape at about a 90° angle. The cup was then filled up with liquid paraffin and closed with a plastic grid cover. The cup was left on a -20 °C plate until the paraffin was solid. The block was then taken out of the cup and stored at room temperature.

3.7.2 Sectioning

Using a microtome, the paraffin-embedded specimen was sectioned at a thickness of 5 µm. The sections were transferred to a water bath at room temperature and subsequently to a water bath at 50 °C to straighten the section. It was then picked up with a SuperFrost Plus glass slide and dried on a heating plate. Finally, the slides were incubated at 60 °C for 1 h and then stored at 4 °C until further use.

3.8 Histochemistry

3.8.1 Safranin O staining

To evaluate the joints for DMM-induced cartilage loss, I stained the sections with safranin O to visualize proteoglycans. In addition, nuclei were stained with hematoxylin and Fast Green was used to obtain a background staining.

The slides were melted at 60 °C for one hour. All staining reagents were filtered. The slides were then deparaffinized in the following way: two times toluene for 10 min each, two times 99 % ethanol for 2 min each, two times 96 % ethanol for 2 min each and two times 70 % ethanol for 2 min each. The slides were then hydrated in deionized water for 2 min followed by staining in Weigert's iron hematoxylin for 10 min. Afterwards, the slides were briefly dipped into 1 % HCl in 70 % ethanol and washed with tap water for 10 min to remove excess staining. The specimens were then stained in 0.05 % Fast Green (FCF) solution for 5 min, followed by a brief dip into 1 % acetic acid solution and staining in 0.1 % Safranin O solution for 20 min. Dehydration of the specimens was achieved by shortly dipping them, two times into 70 % ethanol, two times 96 % ethanol for 2 min, two times 99 % ethanol for 2 min and two times toluene for 2 min. Finally, the specimens were mounted under a cover glass using resinous medium and left for evaporation of the toluene.

3.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism (v5.01). Two-way analysis of variance (ANOVA) analysis with Bonferroni post-test was used to determine statistical significance.

4. Results

4.1 Characterization of osteoclast phenotypes on cartilaginous joint tissues

To shed light on the role osteoclasts play in joint tissues other than bone, I wanted to assess the phenotypic variations they display when grown on these matrices. Therefore, I cultured them in the presence of inhibitors targeting different components of the osteoclastic resorption machinery on articular cartilage and subchondral bone as well as cortical bone as a reference. I assessed their phenotype by measuring their viability, number, parameters of bone and cartilage degradation and the expression of resorption-relevant proteins.

4.1.1 Visual characterization

Figure 5 shows the effect of three different resorption inhibitors on the appearance of resorption pits generated by osteoclasts on cortical bone. The acidification inhibitor diphyltin effectively prevents bone resorption as indicated by the absence of resorption pits. The general MMP inhibitor GM6001 does not affect the resorption pits in a visually detectable way. Inhibition of cathepsins by E64 leads to more shallow pits indicated by a very faint staining.

The staining of resorption pits by hematoxylin is technically not possible on subchondral bone and articular cartilage due to the structural properties of these matrices.

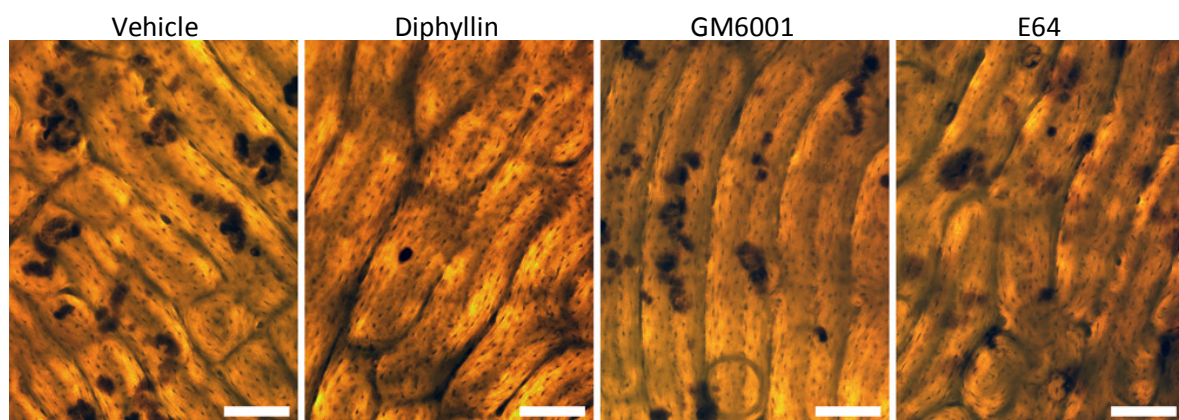


Figure 5. Resorption pit formation on cortical bone in the presence of resorption inhibitors. Mature human osteoclasts were seeded on slices of bovine cortical bone (50,000 cells per well), left to attach for 24 h and cultured in the presence of either diphyltin (200 nM), GM6001 (10 μ M), E64 (5 μ M) or vehicle (DMSO 1:2000) as well as M-CSF and RANKL for 7 days. The resorption pits were visualized by staining of the bone slices with hematoxylin and rubbing off the stain with a cotton swab leaving just the pits stained (dark spots). Scale bars correspond to 200 μ m.

To evaluate the presence of resorbing osteoclasts on all matrices, a co-staining of TRAcP and actin was performed. However, the actin staining on articular cartilage is not shown, because the matrix itself is highly autofluorescent covering any specific staining. As presented in Figure 6, the vehicle condition on cortical bone clearly shows TRAcP-positive multinucleated cells (nuclei indicated by round light spots in the staining) partly surrounded by an actin ring. The TRAcP staining is also present in the inhibitor conditions on cortical bone. The number of cells expressing TRAcP seems increased upon treatment with diphyltin.

However, the actin rings are absent in both the diphyllin and GM6001 condition and they appear very weak in the cells treated with E64. Due to the structure of the subchondral bone slices, it is generally difficult to find the cells, even if they are stained. Thus, the images merely show background staining and sometimes a single cell. Interestingly, however, the cell found in the diphyllin condition on subchondral bone shows TRAcP staining and it appears as if it was surrounded by an intact actin ring. On articular cartilage, no actin staining could be performed, but the TRAcP staining reveals a few osteoclasts in all conditions. However, no significant differences between the conditions can be discerned. Nevertheless, it seems that there are less TRAcP-positive cells on articular cartilage than on cortical bone.

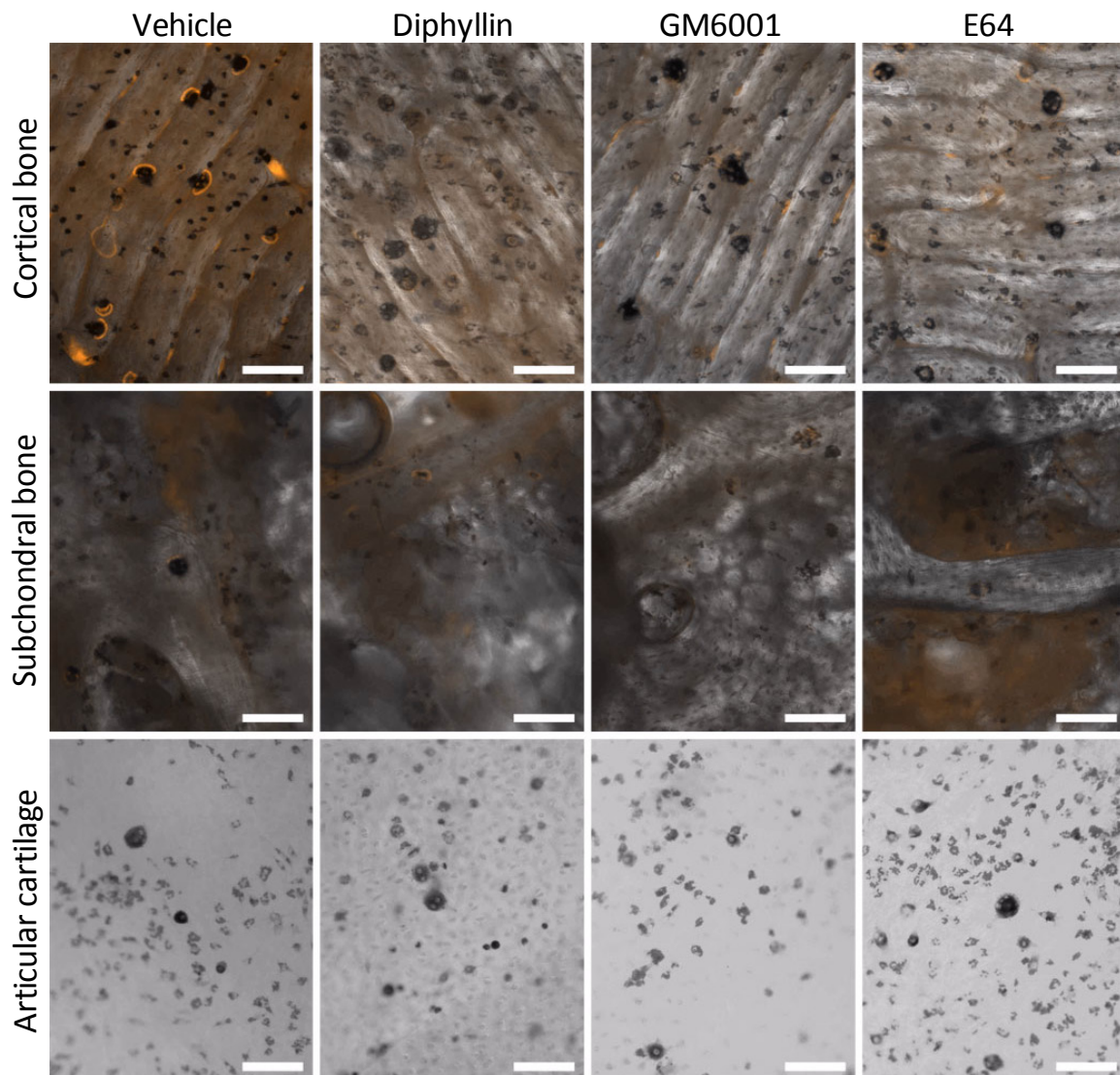


Figure 6. TRAcP / actin co-staining of osteoclasts on different matrices under different inhibitory conditions. Mature human osteoclasts were seeded on slices of bovine cortical bone, subchondral bone and articular cartilage (30,000 cells per well), left to attach for 24 h and cultured in the presence of either diphyllin (200 nM), GM6001 (10 μ M), E64 (5 μ M) or vehicle (DMSO 1:2000) as well as M-CSF and RANKL for 7 days. TRAcP activity was visualized by chromogenic reaction with the Fast Garnet GBC salt (black spots), the cells were then fixed with formaldehyde and actin staining was performed using phalloidin-TRITC (orange staining). The actin staining on articular cartilage was excluded, as it is dominated by the autofluorescence of the matrix. Scale bars correspond to 200 μ m.

4.1.2 Quantification of biochemical markers

To obtain a more quantitative measure of the matrix degrading behavior of the osteoclasts, I measured markers for osteoclast viability, number, calcium release and collagen breakdown in culture supernatants.

To investigate the effect of the matrices on the life span of the cells, the viability was determined by the alamarBlue assay. I found that the resorption inhibitors do not significantly reduce the viability of the cells after 7 days of culture on the different matrices. However, whereas cells on cortical bone are as viable as cells on plastic, the viability on subchondral bone and articular cartilage is reduced (Figure 7A).

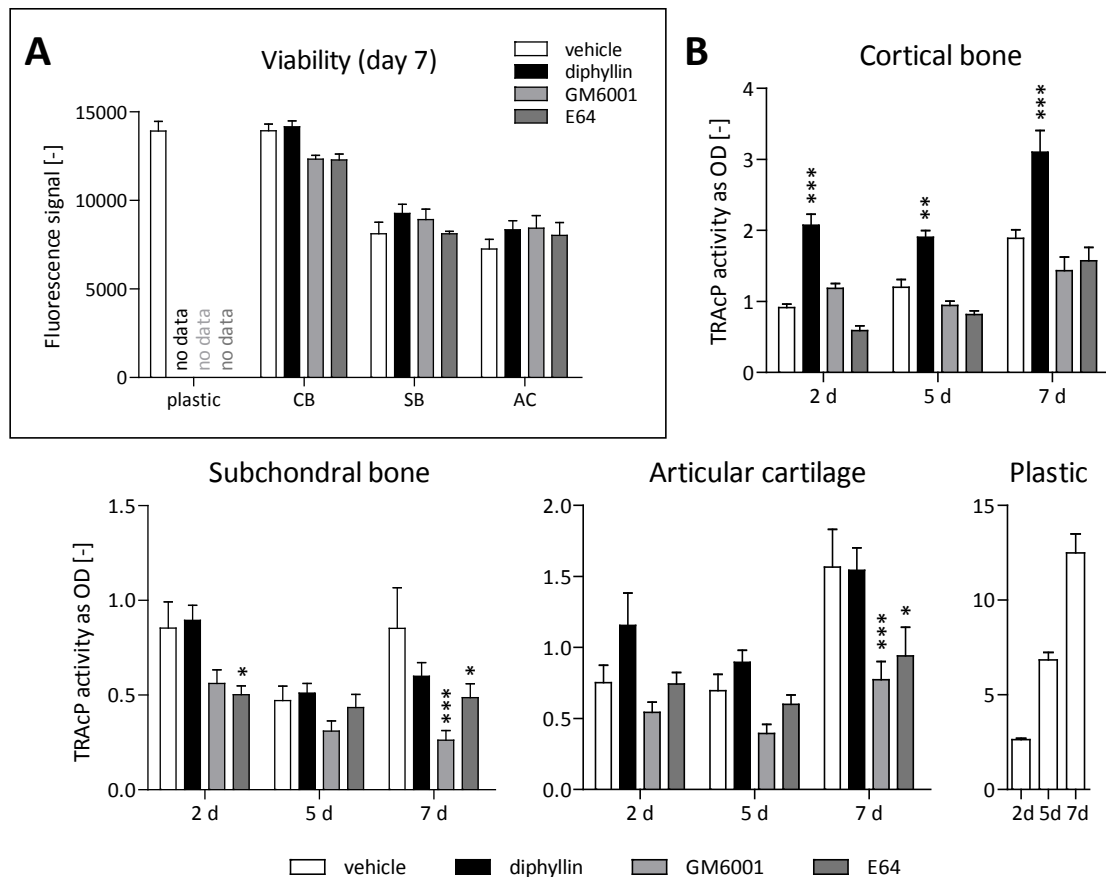


Figure 7. Matrix effect on osteoclast viability and number. Human osteoclasts were generated from CD14-positive monocytes isolated from human peripheral blood by gradient centrifugation and enrichment on magnetic beads. Monocytes were cultured in MEM- α medium containing 10 % fetal bovine serum, 25 ng/ml M-CSF and 25 ng/ml RANKL for 13 days. Osteoclasts were then lifted, reseeded on bovine cortical bone, subchondral bone or articular cartilage slices (50,000 cells per well), left to attach for 24 hours and cultured in the presence of either diphyllin (200 nM), GM6001 (10 μ M), E64 (5 μ M) or vehicle (DMSO 1:2000) as well as M-CSF and RANKL. After 2 and 5 days, supernatants were collected and replaced by fresh dilutions of the inhibitors. The viability of the cells was determined on the final day of the experiment (day 7) by alamarBlue assay (A). TRAcP activity was determined in culture supernatant spectrophotometrically using para-nitrophenylphosphate (B). Plotted values are means of 6 biological replicates corrected for a matrix blank (A & B) and incubation period (B). Error bars indicate $\pm$ SEM. Asterisks mark significant differences between inhibitor and vehicle conditions of the respective time point as determined by 2-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), no indication means non-significant. CB: cortical bone, SB: subchondral bone, AC: articular cartilage.

Further, I determined the TRAcP activity in culture supernatants, to obtain an indication of how the matrices affect osteoclast numbers (Figure 7B). On cortical bone, TRAcP increases slightly over time in the vehicle condition. The GM6001 and E64 conditions do not

deviate significantly from these values, but E64 seems to lag behind somewhat. Diphyllin, on the other hand, increased osteoclast numbers significantly at all timepoints.

On subchondral bone, the pattern is less clear. The protease inhibitors seem to decrease TRAcP release, but significant changes are only found at day 7 (GM6001 and E64) and day 2 (E64). The diphyllin-mediated increase in osteoclast numbers seen on cortical bone is not present on subchondral bone.

The TRAcP levels on articular cartilage resemble those found on subchondral bone, with both protease inhibitors reducing osteoclast numbers significantly after 7 days. Again, diphyllin does not differ significantly from the vehicle condition on articular cartilage.

The reduced viability of osteoclasts on subchondral bone and articular cartilage is reflected in the lower TRAcP values. On plastic osteoclasts were found to release much more TRAcP than on the matrices with a strong increase over time.

Further, markers of bone resorption were measured, namely calcium and CTX-I. As shown in Figure 8A, diphyllin showed a marked reduction in calcium release from cortical bone with an effect enhancing over time. Inhibition of MMPs leads to a significant reduction in extracellular calcium from day 5 on, whereas inhibition of cathepsins shows an effect at day 7.

On subchondral bone as well as on articular cartilage, less calcium was detected and the conditions do not differ significantly for a given time point. On both matrices a steady decrease of extracellular calcium is observed over time.

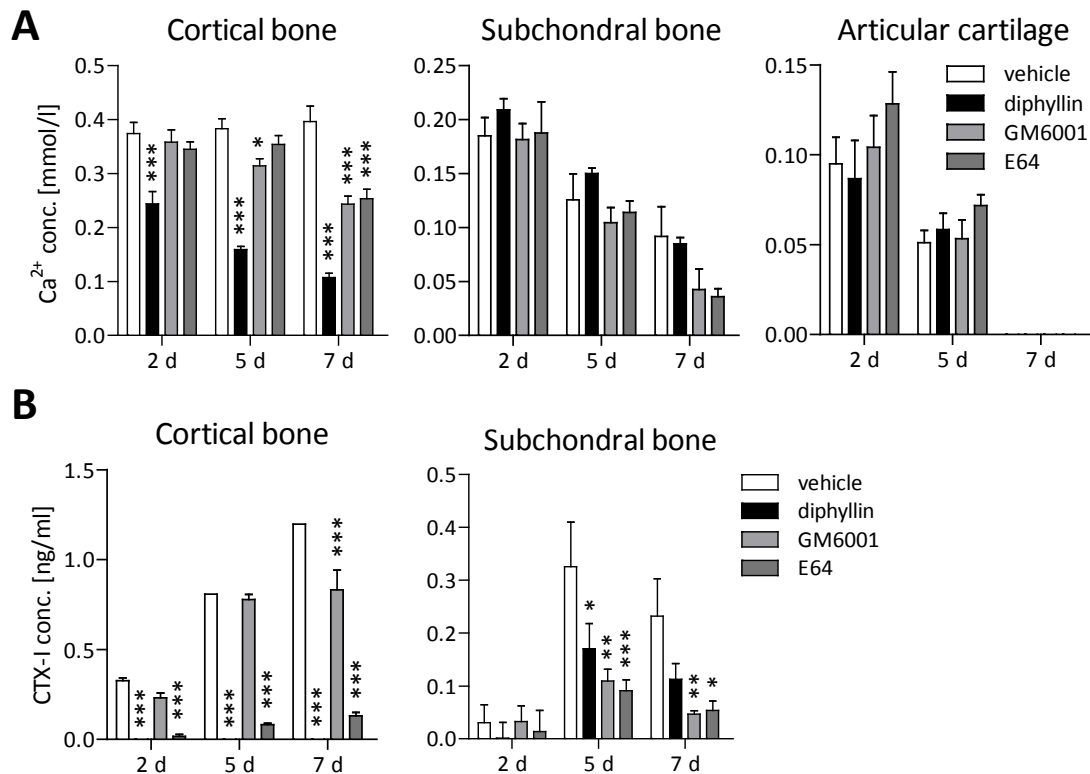


Figure 8. Matrix effect on osteoclastic bone resorption activity. Human osteoclasts were generated from CD14-positive monocytes isolated from human peripheral blood by gradient centrifugation and enrichment on magnetic beads. Monocytes were cultured in MEM- α medium containing 10 % fetal bovine serum, 25 ng/ml M-CSF and 25 ng/ml RANKL for 13 days. Osteoclasts were then lifted, reseeded on bovine cortical bone, subchondral bone or articular cartilage slices (50,000 cells per well), left to attach for 24 hours and cultured in the presence of either diphyllin (200 nM), GM6001 (10 μ M), E64 (5 μ M) or vehicle (DMSO 1:2000) as well as M-CSF and RANKL. After 2 and 5 days, supernatants were collected and replaced by fresh dilutions of the inhibitors. Calcium (A) and CTX-I (B) concentrations were determined in culture supernatant by spectrophotometry and Serum CrossLaps ELISA, respectively. Plotted values are means of 6 biological replicates corrected for a matrix blank and incubation period. Error bars indicate $\pm$ SEM. Asterisks mark significant differences between inhibitor and vehicle conditions of the respective time point as determined by 2-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), no indication means non-significant.

Collagen type I degradation was measured by the release of CTX-I (Figure 8B). Thus, an unambiguous pattern was found on cortical bone. Diphyllin as well as E64 strongly inhibit its release at all time points as expected. The inhibition of MMPs also shows a significant reduction in CTX-I levels at day 7. Due to an oversaturation of the ELISA, the exact CTX-I concentration could not be determined for the vehicle condition at day 5 and 7. It was thus set to the highest value of the standard curve, but might actually be higher. Therefore, a significant effect of GM6001 at day 5 already cannot be excluded. On subchondral bone, no CTX-I is released before day 2, it is detectable at day 5, but then seems decreased at day 7. Its levels are further diminished significantly in the presence of both protease inhibitors. The effect of diphyllin, on the other hand, is much less pronounced than it is on cortical bone. Due to the fact that only minor amounts of collagen type I are found in articular cartilage, no CTX-I concentrations were determined in supernatants of that matrix.

To evaluate the degradation of the primary collagen of cartilage, collagen type II, I measured its degradation products CTX-II and C2M in culture supernatants from

subchondral bone and articular cartilage. Figure 9A shows the CTX-II levels in supernatants from a different experiment, but with the same general setup. The amounts of CTX-II released from subchondral bone are barely detectable. On articular cartilage, CTX-II levels are elevated as compared to the vehicle condition, when the osteoclasts are treated with diphyllin or E64. Moreover, its concentration seems to decrease with time. Nevertheless, the CTX-II concentrations on articular cartilage all lie at the lower end of the standard curve of the ELISA and are, thus, subject to variability.

Figure 9B shows the C2M release from subchondral bone and articular cartilage. On subchondral bone, C2M seems to increase over time in the inhibitor conditions, but remains steady in the vehicle. In the first time point, there is a significant reduction of C2M levels with diphyllin and E64. On articular cartilage, a marked reduction of C2M is seen in the GM6001 treatment, whereas all other conditions show a slight decrease over time but do not differ significantly.

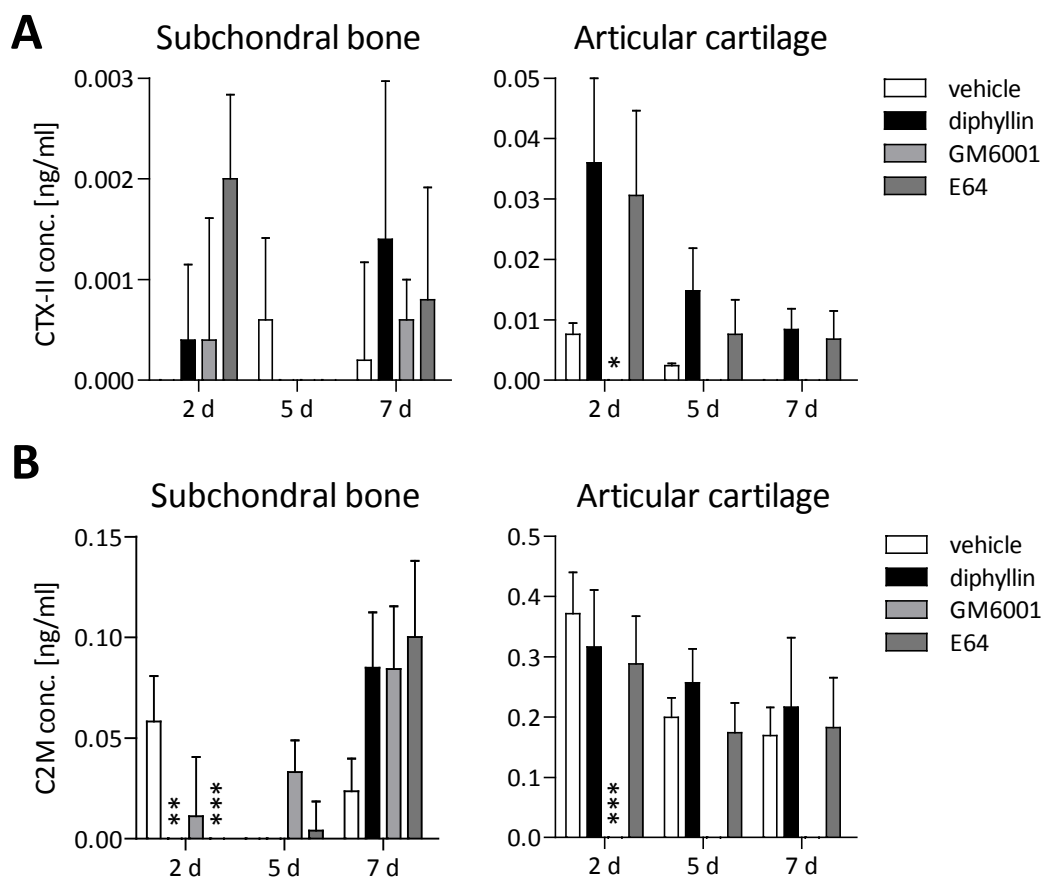


Figure 9. Matrix effect on osteoclastic collagen type II breakdown. Human osteoclasts were generated from CD14-positive monocytes isolated from human peripheral blood by gradient centrifugation and enrichment on magnetic beads. Monocytes were cultured in MEM- α medium containing 10 % fetal bovine serum, 25 ng/ml M-CSF and 25 ng/ml RANKL for 13 days. Osteoclasts were then lifted, reseeded on bovine cortical bone, subchondral bone or articular cartilage slices (30,000 (A) / 50,000 (B) cells per well), left to attach for 24 hours and cultured in the presence of either diphyllin (200 nM), GM6001 (10 μ M), E64 (5 μ M) or vehicle (DMSO 1:2000) as well as M-CSF and RANKL. After 2 and 5 days, supernatants were collected and replaced by fresh dilutions of the inhibitors. CTX-II (A) and C2M (B) concentrations were determined in culture supernatant by ELISAs. Plotted values are means of 5 (A) or 6 (B) biological replicates corrected for a matrix blank and incubation period. Error bars indicate $\pm$ SEM. Asterisks mark significant differences between inhibitor and vehicle conditions of the respective time point as determined by 2-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), no indication means non-significant.

4.1.3 Expression of resorption-relevant proteins

Further, I intended to investigate the expression of key proteins of bone resorption in mature osteoclasts cultured on cortical bone, subchondral bone and articular cartilage for 2, 5 and 7 days, respectively. In addition, I measured TRAcP in the supernatant of these cultures. I, thus, found that the osteoclast activity increases over time on cortical bone as well as on the other two matrices, however to a lesser degree (Figure 10A).

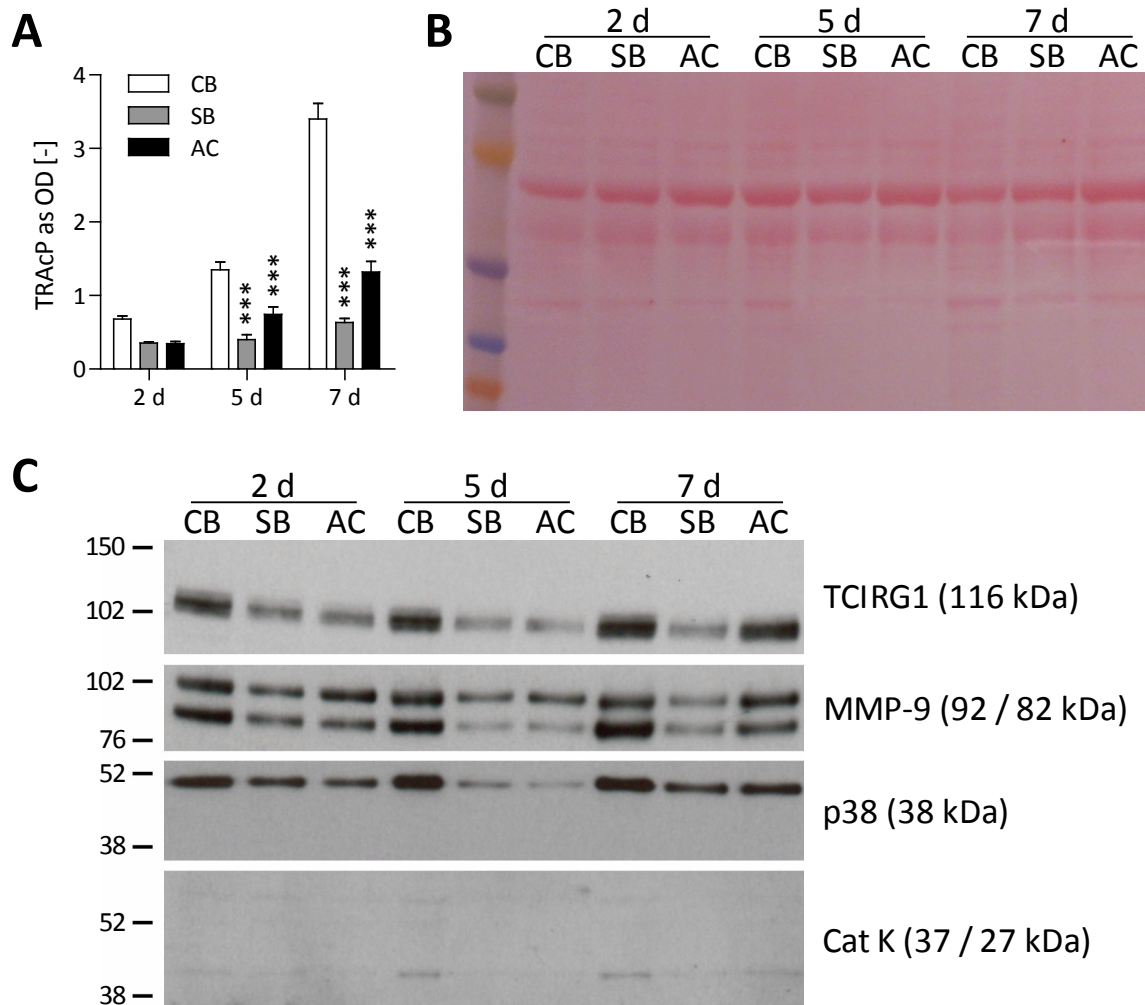


Figure 10. Matrix- and time-dependent changes of osteoclastic protein expression. Human osteoclasts were generated from CD14-positive monocytes isolated from human peripheral blood by gradient centrifugation and enrichment on magnetic beads. Monocytes were cultured in MEM- α medium containing 10 % fetal bovine serum, 25 ng/ml M-CSF and 25 ng/ml RANKL for 12 days. Osteoclasts were then lifted, reseeded on bovine cortical bone, subchondral bone or articular cartilage slices (45,000 cells per well, 20 replicates per time point and matrix) and cultured for 2, 5 and 7 days, respectively. Supernatant was collected and replaced by fresh medium on day 2 and 5. Cells were pooled and lysed using RIPA buffer. 9 μ g of protein were separated by SDS-PAGE, blotted on a membrane and probed for resorption-relevant proteins. A) TRAcP activity in supernatants of 7 day setup as determined by spectrophotometry using para-nitrophenylphosphate. Plotted values are means of 20 biological replicates corrected for background and incubation period. Error bars indicate $\pm$ SEM. Asterisks mark significant differences between SB or AC and CB of the respective time point as determined by 2-way ANOVA (***) $p \leq 0.001$, no indication means non-significant. B) Ponceau staining of protein blotted on the membrane C) Immunological detection of TCIRG1, MMP-9, p38 and Cat K on the membrane. CB: cortical bone, SB: subchondral bone, AC: articular cartilage, TCIRG1: T-cell immune regulator 1, MMP-9: matrix metalloproteinase 9, p38: p38 mitogen-activated protein kinase, Cat K: cathepsin K.

Figure 10B shows the Ponceau staining of the blot membrane which indicates an equal loading of the gel in terms of protein amount. This, however, is not reflected in the amount of the loading control p38 immunologically detected on the membrane (Figure 10C). Therefore, it is difficult to compare the expression of TCIRG1, MMP-9 and Cat K between conditions. What is obvious, though, is that both the V-ATPase subunit TCIRG1 and MMP-9 are expressed at all time points in osteoclasts no matter which matrix they reside on. The Cat K signal, on the other hand, is very weak in all conditions. Moreover, keeping the p38 levels in mind, it seems like the 92 kDa pro-form of MMP-9 is upregulated on articular cartilage at all time points as compared to the other matrices.

4.2 The effect of KBP on osteoclastic bone resorption

In order to evaluate the effect of the novel sCT analog KBP on the bone resorption activity of osteoclasts, I assessed key markers of bone resorption, namely the presence of actin rings as well as the release of calcium and CTX-I in culture supernatant.

4.2.1 Comparison of human and salmon calcitonin

As an initial validation of the system, I wanted to compare KBP's natural competitors, hCT and sCT. Therefore, I compared their anti-resorptive effect, as measured by the release of the collagen fragment CTX-I (Figure 11). A dose-dependent inhibition of collagen degradation is found for both calcitonins at all time points. sCT appears to be more potent than hCT. However, the concentration range examined only seems to cover the linear part of the curve, not the plateaus. Therefore, a shift of the curves due to a technical issue cannot be ruled out. Also worth to mention is that at the earlier time points the lower concentrations of both calcitonins seem to increase the amount of CTX-I released as compared to the vehicle control. On the other hand, again, a technical issue could have caused the reduction of the CTX-I signal in the vehicle control. No detectable amount of CTX-I was found after two hours in all conditions.

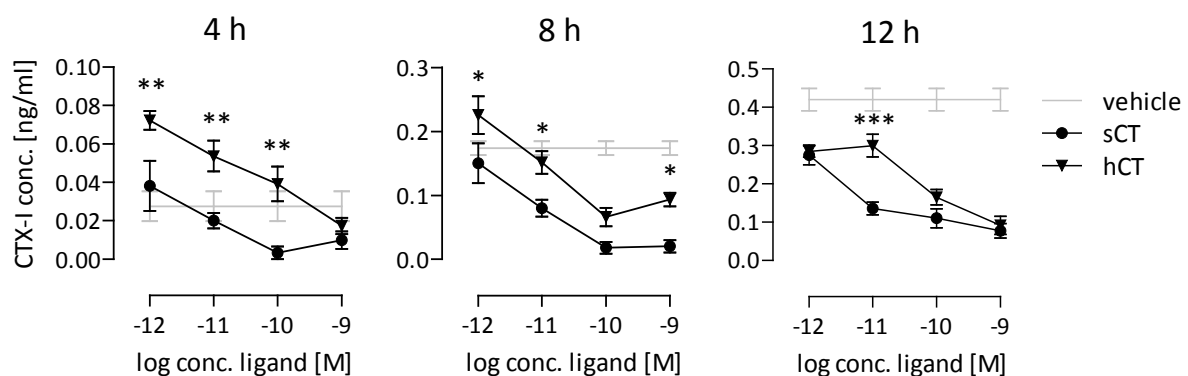


Figure 11. Inhibition of in vitro osteoclastic bone resorption by hCT and sCT. Human osteoclasts were generated from CD14-positive monocytes isolated from human peripheral blood by gradient centrifugation and enrichment on magnetic beads. Monocytes were cultured in MEM- α medium containing 10 % fetal bovine serum, 25 ng/ml M-CSF and 25 ng/ml RANKL for 13 days. Osteoclasts were then lifted, reseeded on bovine cortical bone slices (30,000 cells per well), left to attach for 24 hours and treated with hCT or sCT in different concentrations for 4, 8 and 12 hours, respectively. CTX-I concentrations were determined in culture supernatant by Serum CrossLaps ELISA. Plotted values are means of 6 biological replicates corrected for a matrix blank. Error bars indicate $\pm$ SEM. Asterisks mark significant differences between hCT and sCT conditions as determined by 2-way ANOVA (* p <= 0.05, ** p <= 0.01, *** p <= 0.001), no indication means non-significant.

4.2.2 KBP

Having shown an inhibitory effect of hCT and sCT on bone resorption in my *in vitro* system, I next wanted to assess KBPs potency. First, I compared KBP and the acidification inhibitor diphyllin. Table 1 shows the estimated amount of actin rings observed at different time points. Diphyllin clearly diminishes the number of cells with intact actin rings showing its full effect after 24 hours. KBP also successfully reduces the number of actin rings observed with a more immediate effect than diphyllin. However, a broader concentration range is necessary to confirm a dose-dependent effect.

Table 1. Estimated amount of actin rings upon KBP treatment of osteoclasts compared to diphyllin. The corresponding microscopy images can be found in the appendix (Figure 15).

		2 h	6 h	24 h
Vehicle		+++	+++	+++
Diphyllin		++	+	-
KBP log conc. [M]	-10	-	+	+++
	-9	-	+	+++
	-8	-	+	++

An extension of the concentration range, in a follow-up experiment, showed a difference in the amount of actin rings between the two lowest concentrations of KBP and the three higher concentrations (Table 2).

Table 2. Estimated amount of actin rings upon KBP treatment of osteoclasts. The corresponding microscopy images can be found in the appendix (Figure 16).

		1 h	2 h	4 h	8 h
Vehicle		+++	+++	+++	+++
KBP log conc. [M]	-13	-	-	+	++
	-12	-	+	++	+++
	-11	-	-	-	+
	-10	-	-	-	+
	-9	-	-	+	+

To obtain a more quantitative output from both experiments, I measured the concentrations of calcium and CTX-I released into culture supernatants. In the first experiment, the time-dependent abrogation of actin rings by diphyllin is reflected in the calcium concentrations (Figure 12A). This is not the case for KBP. The CTX-I data, however, show a reduction by both compounds at the 2 and 6 hour time points, with diphyllin showing a higher efficacy.

The second experiment, focusing on earlier time points, shows no effect of KBP after 1 and 2 hours in respect to both calcium and CTX-I release. At the later time points a reduction in both markers is found. CTX-I levels after 8 h indicate a dose-dependent effect of KBP, consistent with the actin rings, separating the two lower concentrations from the three higher ones (Figure 12B).

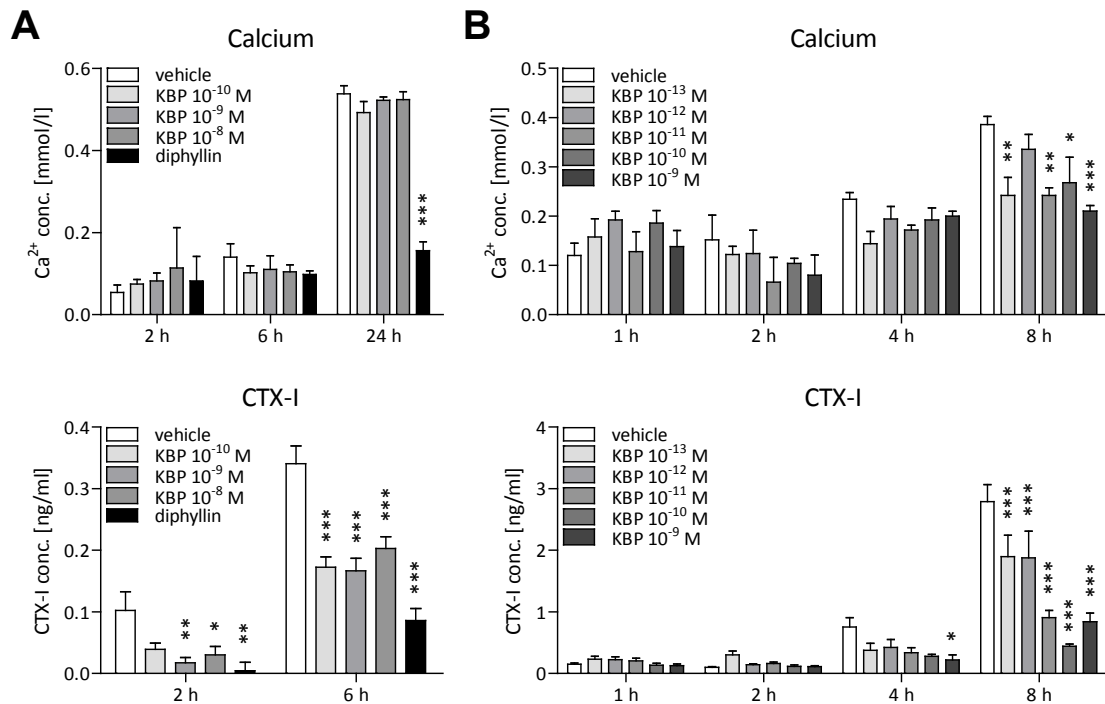


Figure 12. Inhibition of osteoclastic bone resorption by KBP in vitro. Human osteoclasts were generated from CD14-positive monocytes isolated from human peripheral blood by gradient centrifugation and enrichment on magnetic beads. Monocytes were cultured in MEM- α medium containing 10 % fetal bovine serum, 25 ng/ml M-CSF and 25 ng/ml RANKL for 12 days. Osteoclasts were then lifted, reseeded on bovine cortical bone slices (50,000 (A) / 30,000 (B) cells per well), left to attach for 24 hours and treated with different concentrations of KBP for 1, 2, 4, 6, 8 and 24 hours, respectively. Calcium and CTX-I concentrations were determined in culture supernatant by spectrophotometry and Serum CrossLaps ELISA, respectively. Plotted values are means of 5 biological replicates corrected for a matrix blank. Error bars indicate $\pm$ SEM. Asterisks mark significant differences between treated and vehicle conditions of the respective time point as determined by 2-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), no indication means non-significant.

To check whether KBP could outperform its natural equivalent, sCT, I assessed the potential of both to abrogate actin rings and reduce the release of CTX-I. The number of actin rings is clearly diminished in all conditions of the two earlier time points. At the 8 h time point, a concentration-dependent effect is detectable. The data of the 24 h time point is marked by a rather low number of actin rings, even in the vehicle control (Table 3).

Table 3. Estimated amount of actin rings upon KBP treatment of osteoclasts compared to sCT. The corresponding microscopy images can be found in the appendix (Figure 17).

		2 h	4 h	8 h	24 h
Vehicle		+++	+++	+++	+
sCT log conc [M]	-14	+	+	++	+
	-13	+	-	++	+
	-12	+	-	++	++
	-11	-	+	-	+
	-10	-	-	+	++
	-9	+	-	++	++
	-8	+	-	-	+
KBP log conc. [M]	-14	-	-	+	+
	-13	+	-	++	+
	-12	+	+	+	++
	-11	-	+	-	+
	-10	+	-	+	++
	-9	+	-	+	-
	-8	+	-	-	+

The CTX-I data shows a dose-dependent decline in CTX-I levels at 4 and 8 h (Figure 13A). The effect is gone at 24 h. However, the CTX-I levels at 24 h are extremely low compared to the other time points indicating a technical issue. No CTX-I was detectable after 2 h. The CTX-I data does not allow for a differentiation of the potency of both peptides. Therefore, a repetition of the 8 h time point with a higher number of replicates was conducted (Figure 13B). It shows a dose-response curve for both compounds, featuring the linear part as well as the upper and lower plateau. The data does, however, not allow distinguishing both peptides in their potency, but suggests an IC_{50} for both around 0.01 nM.

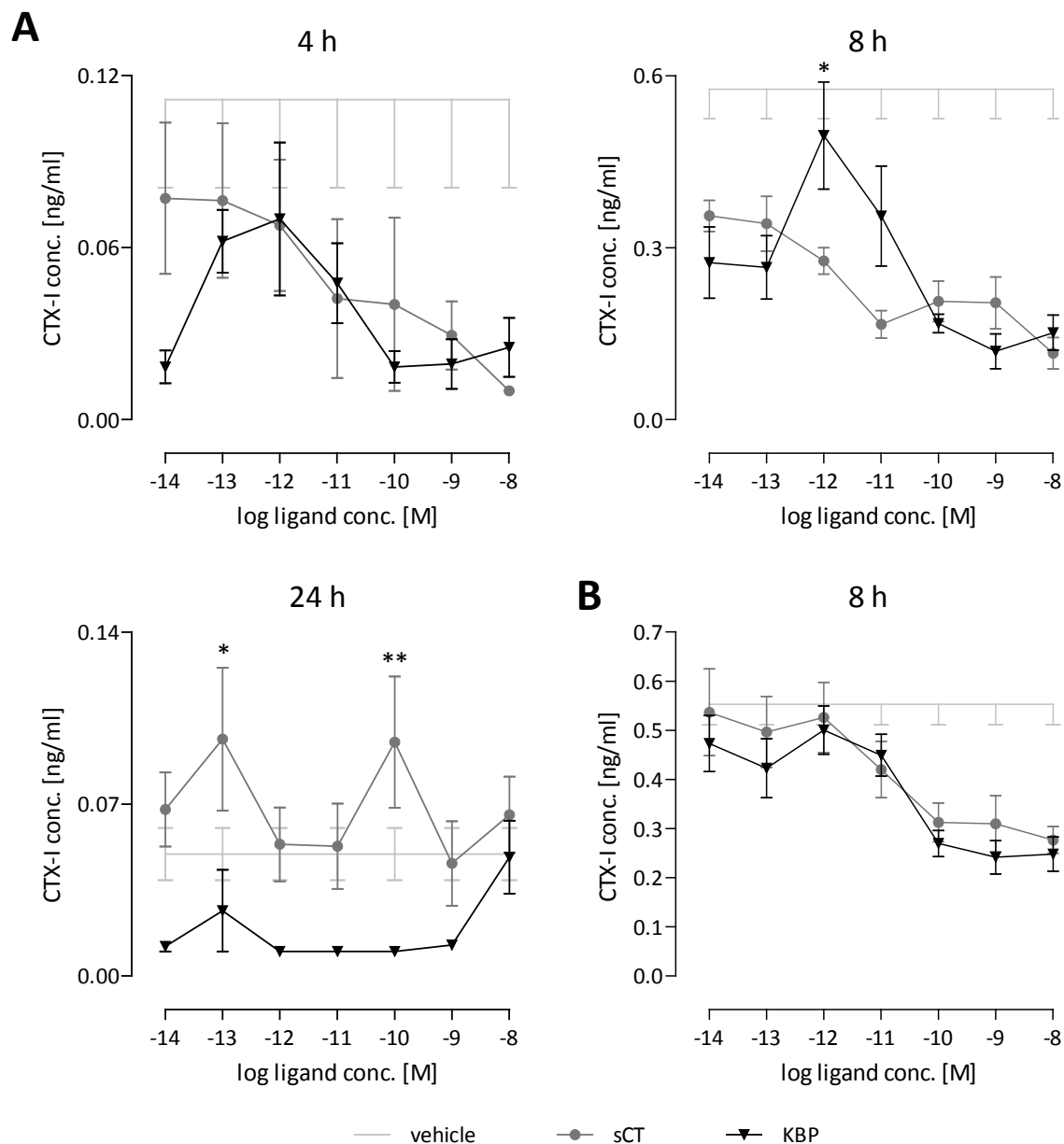


Figure 13. Inhibition of osteoclastic bone resorption by KBP in comparison to sCT. Human osteoclasts were generated from CD14-positive monocytes isolated from human peripheral blood by gradient centrifugation and enrichment on magnetic beads. Monocytes were cultured in MEM- α medium containing 10 % fetal bovine serum, 25 ng/ml M-CSF and 25 ng/ml RANKL for 11 (A) or 12 (B) days. Osteoclasts were then lifted, reseeded on bovine cortical bone slices (30,000 cells per well), left to attach for 24 hours and treated with the respective concentrations of KBP or sCT for 4, 8 or 24 h. CTX-I concentrations were determined in culture supernatant by Serum CrossLaps ELISA. Plotted values are means of 5 (A) or 10 (B) biological replicates corrected for a matrix blank. Error bars indicate $\pm$ SEM. Asterisks mark significant differences between KBP and sCT conditions as determined by 2-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$), no indication means non-significant.

4.3 The resorption-impaired osteoarthritis mouse model

In a mouse model of osteoarthritis with impaired bone resorption, I wanted to determine the osteoclast's involvement in the structural changes of subchondral bone and articular cartilage. To assess the surgical induction of osteoarthritis by DMM, I, histologically, examined the loss of cartilage in both knees, one of which was operated.

Figure 14 shows the safranin O staining of the left and the right knee of a mouse transplanted with control fetal liver cells. One of the knees underwent DMM 6 weeks after the transplantation. Although the integrity of the tissue was somewhat compromised during sectioning, especially in the left knee, a red safranin O staining of articular cartilage as well as the growth plate was obtained. A weakened staining in the cartilage layer covering both the medial femoral condyle and the medial tibial plateau is observed in the left knee (Figure 14B).

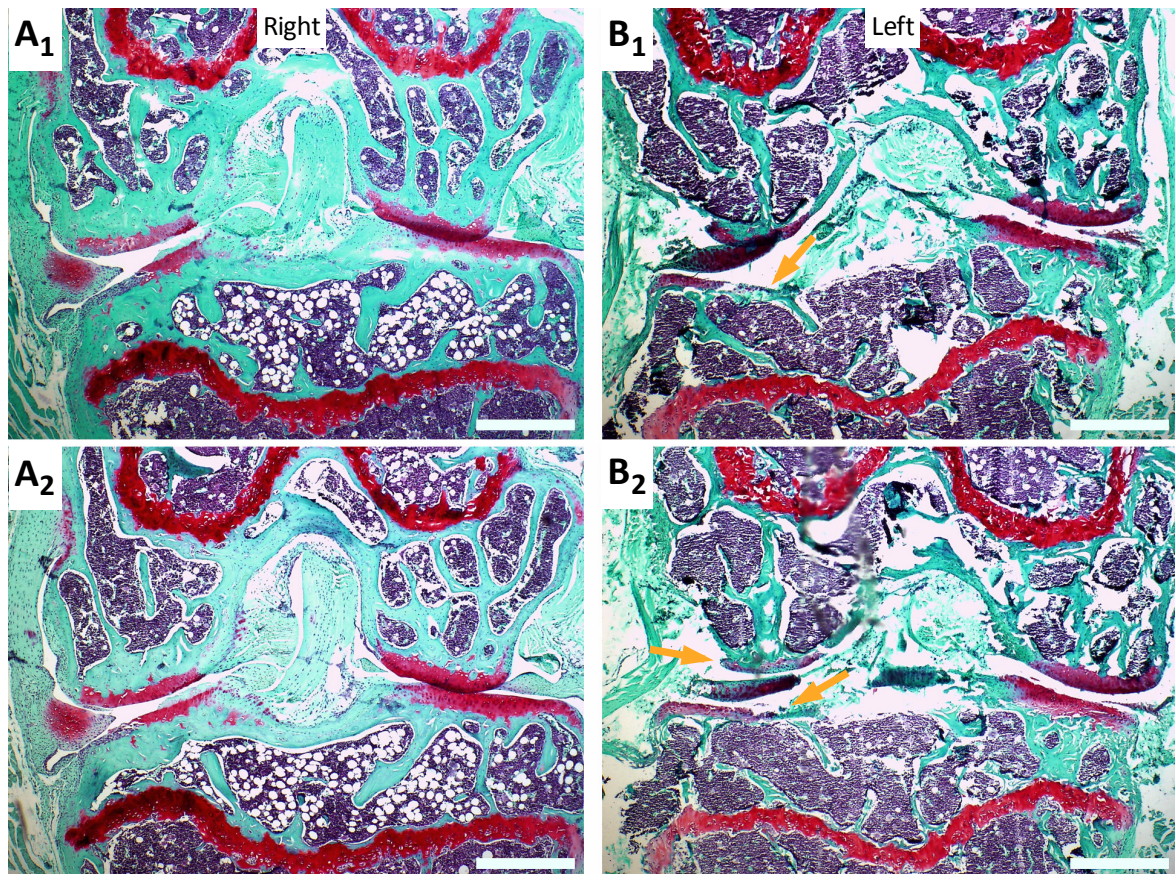


Figure 14. Safranin O staining of both knees of a mouse, one of which underwent DMM. In each image, the femur is to the top, lateral is towards the left (A) or right (B). Orange arrows indicate areas of cartilage loss. Red safranin O staining indicates proteoglycans, nuclei are stained purple and tissue background is green. Scale bars represent 500 µm.

Further evaluation of the model in terms of vascular invasion, ectopic presence of nerves and markers of bone and cartilage degradation, was not possible due to a lack of time.

5. Discussion

5.1 Characterization of osteoclast phenotypes on cartilaginous joint tissues

The remodeling of the subchondral compartment in osteoarthritis is a hallmark of osteoarthritis and likely occurs early in the development of the disease. It includes the loss of trabecular bone, thickening of the subchondral plate, formation of osteophytes and cysts, expansion of the calcified cartilage and duplication of the tidemark (Goldring, 2012). In addition, nerve endings in the osteophytes and a vascularization of the calcified cartilage are observed (Goldring & Goldring, 2007). Given the osteoclast's role in bone remodeling, these findings suggest its involvement in these processes. Further, multinucleated clastic cells on calcified cartilage are called chondrocytes, but phenotypically resemble the osteoclast. They might, in fact, just represent osteoclasts with a shifted phenotype (Wlodarski et al., 2014). The investigation of the osteoclasts potential to adapt to different joint tissues is, thus, of high interest in order to understand what causes the various symptoms of osteoarthritis and to develop a disease-modifying drug. The first part of my thesis is, therefore, dedicated to the *in vitro* study of osteoclast phenotypes on different joint tissues. In particular, I intended to study the suitability of articular and calcified cartilage as a substrate for osteoclasts. Cortical bone, as a native osteoclast matrix, was used as a reference. Due to technical limitations, calcified cartilage is only obtainable as a composite matrix referred to as subchondral bone. It contains undefined amounts of subchondral plate and trabecular bone. This should be kept in mind when interpreting the results.

5.1.1 Inhibitors of osteoclast function

To shed light on possible matrix-dependent differences in the role of some of the key components of osteoclastic bone resorption, I employed small molecule inhibitors to abrogate protein function and subsequently observed phenotypic changes. In particular, I made use of the lysosomal acidification inhibitor diphyllin (Sørensen et al., 2007), the general MMP inhibitor GM6001 (Grobelyny et al., 1992) and the cysteine proteinase inhibitor E64 (Hanada et al., 1978). Assessing their efficacy in preventing osteoclastic resorption of cortical bone, I found changes in the visual phenotype of osteoclasts (Figure 5).

Diphyllin targets the V-ATPase and, thus, abolishes the acidification of lysosomes and the resorption lacuna. Consequently, bone resorption cannot take place, which is in line with the absence of resorption pits I found. The increase in TRAcP-positive cells, indicating enhanced viability of the osteoclasts, has also been described before (Sørensen et al., 2007).

The role of MMPs in osteoclastic matrix degradation is not fully understood so far. It was speculated that they might contribute to collagen degradation under certain circumstances (Delaissé et al., 2003). What is better understood is their involvement in osteoclast migration, exemplified by an increased lifespan of podosomes upon MMP inhibition (Goto et al., 2002). Especially MMP-9 and MMP-14 appear to play a central role in this process (Delaissé et al., 2003). A connection between MMPs and podosomes is also drawn by my findings which showed a disruption of the actin rings upon MMP inhibition. Interestingly, the resorption pits did not differ from those found in the control treatment. The

reason for this remains elusive, as the actin ring is considered to be an essential part of the sealing zone, which is necessary for effective bone resorption.

Cat K is the main enzyme responsible for the degradation of collagen type I in bone. Its absence impairs bone resorption and results in a reduction of resorption pit depth (Goto et al., 2002) which was also reflected in my pit stainings. The attenuation of actin rings I observed upon inhibition of cathepsins has been described before and was attributed to an implication of Cat K in exposing cryptic collagen domains necessary for osteoclast attachment (Wilson et al., 2009).

Technical limitations prevented the visual evaluation of the effect of the inhibitors on osteoclasts on subchondral bone and articular cartilage. Nevertheless, the findings on cortical bone successfully verified the efficacy of the three inhibitors in interfering with key processes of osteoclastic bone resorption *in vitro* and established their use in the study of osteoclast phenotypes in respect to biochemical marker profiles on all three matrices.

5.1.2 Tissue-dependent osteoclast viability and numbers

Before assessing resorption markers, I wanted to evaluate the effect of the matrices on osteoclast viability and numbers. Whereas the inhibitors did not show significant toxicity, both subchondral bone and articular cartilage impaired the viability of mature hOCs, as reflected by the viability assay (Figure 7A). Osteoclast viability is in part regulated by matrix components. The main integrin of osteoclasts, $\alpha\beta3$, has been shown to transmit a death signal in the absence of a ligand (Zhao et al., 2005) or upon binding to the type II collagen N-propeptide (PIIBNP) (Hayashi et al., 2011). PIIBNP is the pro-form of collagen type IIB, the most abundant collagen of cartilage, and might, thus, prevent unintentional degradation of articular cartilage by osteoclasts. Whether PIIBNP was still present in the cartilage specimens I used is unclear and $\alpha\beta3$ ligands, such as vitronectin and fibronectin, are normally present in cartilage (Müller et al., 2014). Further studies are necessary to identify factors which could have caused the reduction in osteoclast viability on subchondral bone and articular cartilage.

The osteoclast-specific TRAcP 5b is frequently used as a marker of osteoclast numbers (Alatalo et al., 2000). This is justified as long as the conditions, such as matrix or inhibitors, do not alter TRAcP expression or half life. The TRAcP values I measured on plastic were considerably higher than on the matrices, which is in accordance with an increased size of the osteoclasts on plastic (data not shown), but does most likely not represent any physiological state (Figure 7B). Further, chondroclasts have been found to secrete less TRAcP (Nordahl et al., 1998). Consequently, TRAcP secretion by osteoclasts cultured on calcified cartilage might, also be reduced independent of osteoclast numbers. Therefore, when interpreting TRAcP values as osteoclast numbers, one should still consider the possibility of changes in osteoclast size and TRAcP expression, in particular on subchondral bone and articular cartilage.

On cortical bone, the inhibition of the acidification of the resorption lacuna by diphyllin resulted in a significant increase of osteoclast numbers (Figure 7B). This was expected, as high levels of calcium were found to constitute a death signal for osteoclasts and inhibiting its release from the bone matrix prolongs the osteoclast's life span (Nielsen et al., 2007). In contrast, a mild reduction in TRAcP activity upon inhibition of cathepsins was observed. This probably does not reflect a reduced activity or number of osteoclasts, but is rather explained by the fact that Cat K cleavage of TRAcP is required for its activation (Ljusberg et al., 2005).

The reduced viability of osteoclasts on subchondral bone and articular cartilage is reflected in the TRAcP values, which are generally a bit lower on these two matrices compared to cortical bone (Figure 7B). However, it might also indicate a change in the osteoclast phenotype, as chondroclasts residing on calcified cartilage secrete less TRAcP (Nordahl et al., 1998). The previously discussed viability-preserving effect of the inhibition of lysosomal acidification is not observed on subchondral bone and articular cartilage. On articular cartilage this might simply be due to a lack of calcium. On calcified cartilage, matrix resorption might work differently than on bone. This is also supported by the fact that chondroclasts barely show a ruffled border (Nordahl et al., 1998). Protease inhibition reduces osteoclast numbers on both matrices with a slightly stronger effect for the inhibition of MMPs. A possible explanation might be the before mentioned involvement of both MMPs and Cat K in osteoclast attachment and the death signal conferred by unoccupied integrin receptors (Delaissé et al., 2003; Wilson et al., 2009; Zhao et al., 2005).

Summarized, the viability and activity of osteoclasts is affected differentially by the joint tissues. Whereas calcium appears to prevent excessive bone resorption on cortical bone through the reduction of osteoclast viability, the resorption of cartilage matrices might be regulated at the point of matrix attachment already.

5.1.3 Parameters of bone resorption

The resorption of bone is marked by the release of calcium and collagen fragments, such as CTX-I. Consequently the inhibition of lysosomal acidification on cortical bone was found to abrogate both calcium and CTX-I release efficiently (Figure 8). Further, the suppression of Cat K-mediated collagen degradation reduced CTX-I release almost completely and showed a delayed decline in extracellular calcium levels which is in accordance with the shallow resorption pits described earlier. Interestingly, MMP inhibition also leads to a mild reduction of both calcium and CTX-I release. The delay of this effect indicates that the involvement of MMPs in matrix attachment is not the primary reason. Instead, the remaining steady increase of CTX-I levels even in the absence of Cat K activity points at a secondary role for MMPs in collagen type I degradation, evidence for which existed before (Henriksen et al., 2006).

On subchondral bone, calcium levels are low and decrease continuously possibly indicating passive release. Further, there is no detectable CTX-I release at day 2. The emergence of CTX-I at day 5 suggests that osteoclasts search the slice for bone matrix, possibly avoiding calcified cartilage. Prevention of migration by inhibition of MMPs, accordingly, reduces CTX-I levels and abrogation of acidification and Cat K activity result in decreased CTX-I release as on cortical bone. This confirms the assumption that the subchondral bone slices are a heterogeneous matrix consisting of both calcified cartilage and bone. This needs to be kept in mind when interpreting results obtained with this matrix.

5.1.4 Osteoclastic degradation of cartilage

The breakdown of cartilage was determined by quantification of collagen type II degradation products. Whereas CTX-II could not be detected on subchondral bone, it was present on articular cartilage where the inhibitors differentially affected its concentration. The inhibition of Cat K increased the CTX-II concentration whereas the inhibition of MMPs caused its complete absence. Interestingly, this has been shown before, not for osteoclasts but for chondrocytes in bovine cartilage explants (Sondergaard et al., 2006a). Furthermore,

blocking of the acidification shows the same effect as the elimination of cathepsin activity. This leads to the conclusion that the MMP-derived CTX-II fragment is further processed by cathepsins which rely on the acidic conditions present in the resorption lacuna and the lysosome.

C2M was released from articular cartilage regardless of inhibition of the acidification or Cat K. In accordance with the notion of C2M being MMP-derived, MMP inhibition completely abolished its signal. This is in line with findings on decalcified cortical bone which indicate a role for MMPs on non-mineralized matrices (Henriksen et al., 2006). In contrast, on subchondral bone, C2M levels increased over time in the absence of MMP activity. After 7 days, C2M also became detectable in the presence of both other inhibitors. Due to the low concentrations, the high variability and the heterogeneity of the matrix, these findings are difficult to interpret. Some compensatory mechanism might exist on subchondral bone.

Taken together, osteoclasts seem to be able to degrade cartilage in a mostly MMP-dependent fashion. Calcified cartilage degradation, on the other hand, appears to require considerable time for the osteoclast to adapt to this substrate. However, how this harmonizes with the decline of calcium release over time remains to be elucidated.

5.1.5 Matrix-dependent expression of resorption-relevant proteins

Furthermore, I investigated phenotypic alterations at the level of protein expression. The results are, however, difficult to interpret due to unequal protein loading on the gel as indicated by fluctuating p38 levels. The rather uniform loading of protein apparent in the Ponceau staining of the membrane suggests that this is caused by considerable amounts of protein released from the matrix which strongly influence the initial normalization for protein concentrations of the lysates. Attempts for a more gentle, biochemical detachment of the osteoclasts by the integrin ligand echistatin, as demonstrated before (Wesolowski et al., 1995), failed. This is possibly due to the remaining interaction of CD44 with the matrix.

The relative increase of pro-MMP-9 in osteoclasts on articular cartilage would support the previously discussed role for MMPs in osteoclastic cartilage degradation. However, the very low level of Cat K, a key osteoclast marker, in all conditions requires caution when interpreting these findings. Macrophages as a source of altered protein levels cannot be excluded.

In short, for the examination of protein expression changes a method needs to be established which allows lysing osteoclasts without releasing protein from the matrices.

5.1.6 Limitations

Osteoclasts are terminally differentiated cells, hence cannot be kept in culture as a cell line. Therefore, they have to be generated fresh from donors for individual experiments. Since recombinant RANKL has become commercially available, osteoclasts can be generated in large quantities *in vitro* from monocyte precursors obtained from human peripheral blood (Sørensen et al., 2006). Although this has a number of advantages, such as a high yield, a relative longevity of the osteoclasts and a homogeneous cell population with almost complete absence of other cell types, it is still a challenging and time-consuming procedure; obtaining multinucleated, mature osteoclasts from blood takes about 12 days. Moreover, the yield is quite variable due to great variances in the osteoclastogenic potential of blood from different individuals (Durand et al., 2011) as well as a sensitivity of the precursors to several factors, such as cell density (Schilling et al., 2004) and characteristics

of the bovine serum supplement. Therefore, repetitions of the same experiment with osteoclasts from different donors can show divergent results. The use of buffycoats pooled from four donors, as those which were used in the experiments presented here, can ameliorate this, but only in a limited way.

Another technical challenge is the preparation of the matrices in a consistent fashion. While a well established procedure for the production of cortical bone slices for the use in microwell-based *in vitro* experiments exists, this is not the case for subchondral bone and articular cartilage. Cartilage was obtained by punch biopsy from the femoral condyle. This comes with some problems, such as irregularities in the shape due to the flexibility of cartilage and a possible presence of calcified cartilage. Especially the bending of the slices and their irregular shape poses a problem when seeding cells on the matrix. Thus, an undefined amount of osteoclasts might actually settle on the plastic around and beneath the cartilage slice, exhibiting a different phenotype and, thus, a source of error. Subchondral bone, like cortical bone, is drilled out of the bone as a stick which can then be sliced. During this process, it is not possible to differentiate between calcified cartilage and the underlying bone. Inconsistencies in the composition of this matrix are, therefore, possible. Further, the fragility of subchondral bone makes its handling a delicate task and holes in the slice can sometimes appear, giving the cells way to the plastic surface.

Of course, limitations in regard to the *in vivo* significance of results obtained with this model exist as well. For instance, one has to keep in mind that human osteoclasts might behave differently on matrices from bovine origin than they would on the respective human tissue. Furthermore, the lack of other cell types and tissues in this model and, thus, the absence of positive or negative regulatory mechanisms needs to be considered.

Taken together, the combination of both the phenotypically inconsistent osteoclast precursors and the poorly established generation of assay-suitable matrices of calcified and articular cartilage make the *in vitro* study of osteoclast phenotypes on these matrices a tricky endeavor. The improvement and standardization of these procedures is, therefore, highly aspired.

5.2 The effect of KBP on osteoclastic bone resorption

The inhibitory effect of sCT on bone resorption has long been exploited to treat skeletal diseases. Due to cartilage-protective indications, it was recently evaluate as a drug for osteoarthritis. A limited efficacy in clinical trials, however, prevented its application as a drug so far. Adjustment of pharmacological parameters, such as the formulation and a better characterization of patient subpopulations might change this eventually. Nevertheless, alongside these pharmacological attempts to pave the way for the clinical application of sCT, derivatives with a higher potency are sought. In the second part of my thesis, I wanted to evaluate the potency of the sCT analog KBP in inhibiting osteoclastic bone resorption *in vitro*. Therefore, I compared its efficacy in modulating biochemical and cytochemical markers of bone resorption in comparison to the acidification inhibitor diphyllin and sCT.

5.2.1 Influence on functional parameters of bone resorption

An initial comparison of sCT to its human homolog points at an increased potential of sCT to prevent bone resorption, measured by CTX-I release. This is in accordance with previous findings (Sturtridge & Kumar, 1968) and suggests that my *in vitro* model of bone resorption is suitable for the comparison of KBP with other inhibitors. However, differences in

earlier time points might be obscured by technical inconsistencies, as indicated by the shift of the vehicle control relative to the calcitonin conditions over time.

KBP shows an immediate effect in the disintegration of actin rings (Table 1). Although the exact mechanism of sCT is not clear, evidence indicates that it impairs the motility of osteoclasts (Chambers & Magnus, 1982). This would be well aligned with the fast dissolution of the actin rings. In contrast to diphyllin, however, the effect subsides within 24 h as indicated by actin rings and extracellular calcium levels (Figure 12). The calcium assay is probably not sensitive enough to detect differences in calcium concentrations at the earlier time points. The more sensitive CTX-I ELISA, on the other hand, revealed a resorption-preventive effect of KBP already after 2 h and, with a broader concentration range, a dose-dependent effect (Figure 12). The dose-dependent effect is also supported by the actin staining (Table 2). The higher potency of diphyllin in reducing CTX-I levels might be explained by its more direct effect on collagen degradation through the inhibition of the acidification of the resorption lacuna. This impairs the demineralization of the collagen matrix and prevents an optimal pH for Cat K.

A direct comparison of KBP with sCT showed a dose-dependent effect in the reduction of actin rings and CTX-I levels (Table 3 and Figure 13) for both peptides at 2, 4 and 8 h. The reason for the low number of actin rings in the vehicle control and the low CTX-I levels after 24 h remains elusive. Repeating the 8 h time point with more replicates, I found a dose-response effect which did not differ significantly between KBP and sCT. According to the before mentioned differences between high and low concentrations of KBP, the dose-response curve points at an IC_{50} around 0.01 nM.

To sum up, KBP is capable to reduce bone resorption in subnanomolar concentrations *in vitro*, possibly through the inhibition of osteoclast motility. It shows an immediate effect, visible after 2 h and measurable after 4 h which subsides within 24 h. Kinetically it is not superior to sCT in inhibiting bone resorption.

5.3 In vivo study of the role of osteoclasts in osteoarthritis

Osteoarthritis is marked by changes in all tissues of the joint. The mechanical interaction between these tissues is most likely not the only reason for this. In fact, a complex crosstalk between the cells present in the joint through cytokines and the release of extracellular matrix components is likely. Of particular interest is the connection between changes in the subchondral bone and the articular cartilage, both occurring early in the pathogenesis of osteoarthritis. Whereas *in vitro* studies are suitable to narrow in on a specific molecular process while eliminating effects from peripheral sources, only *in vivo* models provide the natural context required to investigate the molecular crosstalk on the tissue and organ level. In the third part of my thesis, I intended to investigate how osteoclasts contribute to the structural changes in the subchondral bone and articular cartilage observed in osteoarthritis.

5.3.1 Findings from previous animal models

The role of osteoclasts in osteoarthritis has been studied *in vivo* before. Hayami et al. and Siebelt et al. demonstrated that bisphosphonate treatment of rats rendered osteoarthritic by anterior cruciate ligament transection (ACLT) or papain injection combined with running exercise reduces subchondral bone remodelling (Hayami et al., 2004; Siebelt et al., 2014). Whether this can solely be attributed to the inhibition of osteoclast function remains to be

determined as bisphosphonates can also affect other cells of the monocyte lineage (Roelofs et al., 2010). Further, bisphosphonates induce apoptosis in osteoclasts and thus impair the osteoclast-dependent activity of osteoblasts. This is of importance, as a cross-talk between osteoblasts in the subchondral compartment and chondrocytes in the articular cartilage might exist and contribute to the pathological changes in both tissues (Findlay & Atkins, 2014). Instead of depleting osteoclasts, another study aimed at merely inhibiting bone resorption by abrogating Cat K function in ACLT models of rabbits and mice. Both models showed reduced cartilage damage (Hayami et al., 2012). These findings indicate that osteoclasts participate in the pathological changes of osteoarthritis. This might include the vascular and neuronal invasion of the subchondral bone, both processes which rely on remodeling activities. Especially the growth of sensory neurons in the subchondral bone might have direct implications as a primary source of pain in osteoarthritis (Suri et al., 2007). The osteoclast, therefore, represents a possible target for future disease-modifying drugs.

5.3.2 Description and aim of our model

Recently, an osteopetrosis mouse model characterized by either dysfunctional osteoclasts or abrogated osteoclastogenesis was used by Thudium et al. to study the osteoclast's role in bone remodeling (Thudium et al., 2014). We now intended to take the model one step further and study the role of osteoclasts in osteoarthritis with a focus on cartilage loss and increased vascularization and innervation of the subchondral bone. As done previously, we substituted the hematopoietic system of healthy mice, aged 3 months or older, with that of an osteopetrotic phenotype. To account for the coupling, we included both the osteoclast-rich and the osteoclast-poor subtype, characterized by dysfunctional osteoclasts (TCIRG1^{-/-}) or a lack in the capability to form osteoclasts (RANK^{-/-}), respectively (Thudium et al., 2014). In addition, osteoarthritis was then induced surgically by DMM (Glasson et al., 2007). Subsequently, we intended to quantify the loss of subchondral bone and articular cartilage histologically and through biochemical serum markers. Further, we wanted to evaluate the presence of nerves and blood vessels in the subchondral compartment by immunohistochemistry.

5.3.3 Cartilage damage induced by DMM

Unfortunately, a lack of time prevented me from a comprehensive study of the experiment. What I managed to do was the histological examination of the effect of the DMM surgery in a control mouse. Blindly assessing the staining, I recognized a loss of safranin O staining in the left knee (Figure 14B). This would suggest a positive impairment of articular cartilage by the DMM surgery. However, it turned out that the right knee was the operated one. Reasons for this disagreement might be a shift of loading towards the unoperated knee and, thus, increased abrasion or a difference in the spatial orientation of both knees during sectioning. Embedding and sectioning of the knees requires practice and time. The knees of one mouse are not sufficient to conclude anything in regard to whether the model worked or not. It is, therefore, necessary to examine the remaining control knees to validate the model before a histological characterization of the different genotypes can take place.

In summary, our model might provide new insights in the participation of osteoclasts in the vascularization and innervation of the subchondral bone as well as articular cartilage loss in osteoarthritis while considering the coupling of osteoclasts and osteoblasts. Further time-

consuming analysis of specimens to, first, validate and then characterize the model is, however, necessary.

5.3.4 Limitations

In vivo models of osteoarthritis are a helpful tool for studying factors involved in the progression of the disease and assessing potential drugs. The central aim thereby is to obtain a predictive model for the situation in humans. The fact that no disease-modifying drug for osteoarthritis has entered the market so far shows that this still poses a challenge. Choosing a model is usually a compromise. On the one hand, it should be sensitive, predictive and resembling the human physiology as much as possible. On the other hand, the model needs to be accessible, technically feasible and suited for the question to be addressed. When drawing conclusions from an *in vivo* osteoarthritis model, key parameters such as species, age and disease induction technique should be kept in mind.

Given the availability of transgenic strains and the relative ease in handling, we used a mouse model. However, studying a disease predominantly occurring in aged individuals in a rodent model requires compromises regarding the age of the animals used. Too young animals are not suitable as ongoing skeletal morphogenesis might distort disease processes. On the other hand, obtaining aged mice takes much longer and they are more likely to develop additional diseases, such as tumors. We decided to transplant the mice when they were at least 3 months old. As long bone morphology in C57BL/6 mice has been shown to approach maturity at 12 weeks of age, this was considered a reasonable compromise (Ferguson et al., 2003). However, it is worth to mention that for instance the post-menopausal drop in sex hormone levels affecting osteoclasts, especially implicated in osteoporosis, is disregarded in this model due to the age of the animals.

The transplantation efficiency represents a critical factor in this experiment. Given that heterozygotes (TCIRG1^{+/-} and RANK^{+/-}) are haplosufficient, already a small amount of remaining wild type hematopoietic stem cells might render the transplantation inefficacious. While engraftment levels of 98 % can be achieved (Henriksen et al., 2011), in this experiment only 93 % were reached. Whether this is still sufficient to deplete osteoclasts or their function needs to be assessed by TRAcP and CTX-I concentrations in serum samples.

ACLT is widely used in bigger animals, but in mice more difficult to perform. It is, further, a relatively severe model for osteoarthritis resembling the later stages of the disease (Glasson et al., 2007). Subchondral bone remodeling is thought to occur at early stages of the pathogenesis. How this influences cartilage pathology and *vice versa* and which tissue is affected first is unclear. In an ACLT model, cells might, therefore, behave differently than in a slowly progressing idiopathic form of osteoarthritis. The DMM technique, in comparison, is a more mild model, merely affecting articular cartilage (Glasson et al., 2007). Thus, it is perhaps less affected by harsh biomechanical alterations which might conceal the effects assessed in the model. Therefore, DMM should be well suited for the study of the contribution of osteoclasts in osteoarthritis. Nevertheless, technically it is a very delicate operation and inconsistencies in the severity of the effect should be expected.

Lastly, the consistent histological preparation of specimens holds some challenges. For instance, the angle between femur and tibia and the orientation of the joint in the paraffin block can vary between knees. This might result in tissue compartments appearing bigger or smaller and, thus, in an over- or under-estimation of cartilage thickness.

5.4 Conclusion and perspectives

During my thesis, I studied the osteoclast's involvement in the structural changes of cartilaginous tissues occurring in osteoarthritis *in vitro* and *in vivo* as well as the potency of inhibiting its function with KBP.

My findings suggest that osteoclasts exert a catabolic role on cartilage tissues based on MMPs and adapt their phenotype accordingly. This points at a role for osteoclasts beyond bone and might represent a potential target mechanism for future disease-modifying drugs for osteoarthritis. A more homogenous matrix of calcified cartilage would, however, be necessary to study this in more detail. Further, it needs to be determined in how far macrophages contribute to cartilage degradation. Future studies, therefore, should focus on establishing a procedure to obtain microwell-suited matrices for calcified cartilage and possibly even trabecular bone as an alternative reference matrix for osteoclastic bone resorption. The involvement of macrophages in cartilage degradation could be evaluated by culturing osteoclast-free macrophage cultures on the cartilaginous matrices and comparing the resorption marker profile to that of osteoclasts. Moreover, the examination of protein expression profiles of osteoclasts cultured on different joint tissues could yield indications about the relevance of the acidification machinery and the role of specific MMPs in the matrix-dependent phenotypic changes. This, however, would require a method to obtain osteoclast lysates free of matrix protein. Lastly, it is of great interest to identify the factors on calcified and articular cartilage which regulate osteoclast viability and adaptation.

Testing the novel sCT analog KBP, I showed its potential to inhibit osteoclastic bone resorption *in vitro*. However, my findings do not indicate superiority over sCT. Whether potential differences are just covered by the limitations of the *in vitro* system remains to be determined. In addition, further studies are necessary to dissect whether additional benefits of sCT, such as the analgesic effect and cartilage protective and anabolic effects, are also provided by KBP. Apart from osteoclast culture experiments on cartilage matrices, this would require *in vivo* studies. Additionally, elucidating differences in downstream signaling between hCT and sCT could provide a better understanding of the increased potency of teleost calcitonins and support the design of more efficacious derivatives.

Lastly, I began to evaluate the induction of osteoarthritis by DMM in a mouse model of osteoarthritis with impaired bone resorption. An adequate validation of the mouse model requires the examination of knees from more mice as well as the measurement of osteoclast serum markers, such as TRAcP and CTX-I. If the transplantation and the DMM surgery were successful, this model might provide new insights in the involvement of osteoclasts in osteoarthritis progression. In particular, it might provide answers to whether the direct signaling of osteoclasts to other cells of the bone compartment plays a role in the structural changes of the subchondral bone and the articular cartilage.

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7. Appendix

7.1 Materials

7.1.1 Chemicals

	Brand	Catalog no.
2-mercaptoethanol	Sigma	M6250
4-Nitrophenyl phosphate disodium salt hexahydrate	Sigma	N4645
Acetic acid (glacial) 100 %	Merck	1.00063
alarmarBlue reagent	Thermo Scientific	88952
Bronidox L5	Cognis	4505205
CD14 Dynabeads	Invitrogen (Life Technologies)	11149D
Deoxycholic acid	Sigma	D6750
Dimethyl sulfoxide	Sigma	D2650
Diphyllin	Asinex	BAS 00442101
E64	Sigma	E3132
Ethanol absolut TechniSolv ren 99.5 %	VWR	C83813.360
Ethylenediaminetetraacetic acid disodium salt dihydrate	Sigma	E5134
Fast Green FCF	Sigma	F7252
Fetal Bovine Serum	Sigma	F7524
Ficoll-Paque PLUS	GE Healthcare	17-1440-03
Formaldehyde solution 4 %	Merck	1.00496.5000
GM6001	Sigma	M5939
Hematoxylin	Sigma	H3136
Human calcitonin trifluoro acetate salt	Bachem	4014409.0001
Hydrochloric acid >=37 %	Sigma	30721N
IGEPAL CA-630	Sigma	I3021
Iron(III) chloride hexahydrate	Sigma	F2877
KBP-056		
L-Ascorbic acid	Sigma	A0278
M-CSF	R&D	216-MC
MEM alpha	gibco (Life Technologies)	AS32571
Nitrogen (liquid)		
NuPage LDS sample buffer (4x)	Life Technologies	NP0008
NuPage MOPS SDS running buffer (20x)	Life Technologies	NP0001
NuPage reducing agent (10x)	Life Technologies	NP0004
Paraffin Paraplast Plus	Sigma	P3683
Penicillin-Streptomycin	Sigma	P4333
Phalloidin-TRITC	Sigma	P1951
Phosphate buffered saline	Sigma	D8537
Ponceau S solution	Sigma	P7170
Protease inhibitor Complete, EDTA-free	Roche	5056489001
Rainbow marker, full length	GE-Amersham	RPN800E
RANKL	R&D	390-TN
Safranin O	Sigma	S2255
Salmon calcitonin	Bachem	H2260
Skim milk powder	Fluka	70166
Sodium chloride	Sigma	S3014
Sodium dodecyl sulfate	Sigma	L4390
Sodium hydroxide	Merck	1.06498
Sodium tartrate dihydrate	Merck	1.06664.0100
Thymidine	Appllichem	A2265

	Brand	Catalog no.
Toluene		
Tris base	Sigma	T1503
Tris hydrochloride	Sigma	T3253
Triton X-100	Sigma	9002-93-1
Trypsin-EDTA solution	Sigma	T3924
Tween 20	Merck	822184
Western Blot Detection Reagents ECL	GE Amersham	RPN2106

7.1.2 Antibodies

	Brand	Catalog no.
Cathepsin K rabbit	Abcam	ab85716
Goat anti-rabbit HRP-conj.	Jackson ImmunoResearch	111-035-003
MMP-9 mouse mAb	Calbiochem	IM37
p38 MAPK rabbit	Cell Signaling	9212
Rabbit anti-mouse HRP-conj.	Jackson ImmunoResearch	315-035-045
TCIRG1 mouse mAb	Abnova	H00010312-M01A

7.1.3 Kits

	Brand	Catalog no.
C2M ELISA	Nordic Bioscience	-
CTX-I ELISA	Nordic Bioscience	4CRL4000
DC protein assay kit II	Bio-Rad	500-0112
Leukocyte acid phosphatase (TRAP) kit	Sigma	387A-1KT

7.1.4 Supplies

	Brand	Catalog no.
Assay plate 96 well	Brand	781602
Assay plate 96 well, black	Costar	3915
Cell culture flask (150cm ²)	Corning	430825
Cell culture flask (75 cm ²)	Corning	430641
Cell culture plate 96 well	Almeco	92096
Conical tubes (15 ml)	Corning	430790
Conical tubes (50 ml)	Corning	430828
Hyperfilm ECL	GE Healthcare	28-9068-35
iBlot gel transfer stacks, nitro cellulose	Life Technologies	IB301002
Magnetic particle concentrator	Dynal Biotech	MPC-1
NuPage Novex 4-12% Bis-Tris protein gel	Life Technologies	NP0321
SuperFrost Plus glass slide	VWR	48311-703

7.1.5 Devices

	Brand	Model
Centrifuge	Eppendorf	5810
iBlot dry blotting system	Life Technologies	IB1001
Incubator for cell culture	Nuaire	DH Autoflow
Microscope, inverted	Olympus	IX70
Microtome	Micron	HM360
Minitom	Struers	04436133
Sonication bath	Buch & Holm	Bandelin Sonorex
Spectrophotometer	Molecular devices	SpectraMax M5
UV oven	Stratagene	Stratalinker 2400

7.1.6 Buffer and media recipes

RANKL-negative hOC medium:

500 ml	MEM alpha
60 ml	FBS
6 ml	penicillin-streptomycin
1100 µl	thymidine
25 ng/ml	M-CSF

RANKL-positive hOC medium:

567 ml	RANKL-negative hOC medium
25 ng/ml	RANKL

RIPA buffer

600 µl	sodium chloride 5 M
5 ml	Tris hydrochloride 1 M, pH 7.4
1 ml	EDTA 0.5 M, pH 7.4
1 g	IGEPAL
1 g	deoxycholic acid
1 ml	sodium dodecyl sulfate 10 %
Add deionized water up to 100 ml.	

TBST (50 x)

154.4 g	Tris base
149.0 g	sodium chloride
16.7 ml	Bronidox L5
56.2 g	Tween 20
700 ml	deionized water
Adjust to pH 7.2 with hydrochloric acid.	
Add deionized water up to 1000 ml.	

TRAcP reaction buffer:

59 ml	acetic acid (glacial) 100 %
5 ml	Triton X-100
58 g	sodium chloride
3.72 g	EDTA
700 ml	deionized water
Adjust to pH 5.5 with sodium hydroxide.	
Add deionized water up to 1000 ml.	

TRAcP solution buffer:

1 ml	8.8 mg/ml L-ascorbic acid (freshly prepared)
1 ml	46 mg/ml sodium tartrate dihydrate (freshly prepared)
1 ml	18 mg/ml 4-nitrophenyl phosphate (freshly prepared)
2 ml	TRAcP reaction buffer
3 ml	deionized water

Weigert's iron hematoxylin:

1.16 g	iron(III) chloride hexahydrate
99 ml	deionized water
1 ml	hydrochloric acid 37 %
100 ml	1 % hematoxylin in 95 % ethanol

7.2 Actin stainings

7.2.1 Actin staining of KBP (0.1 – 10 nM) vs. diphyllin

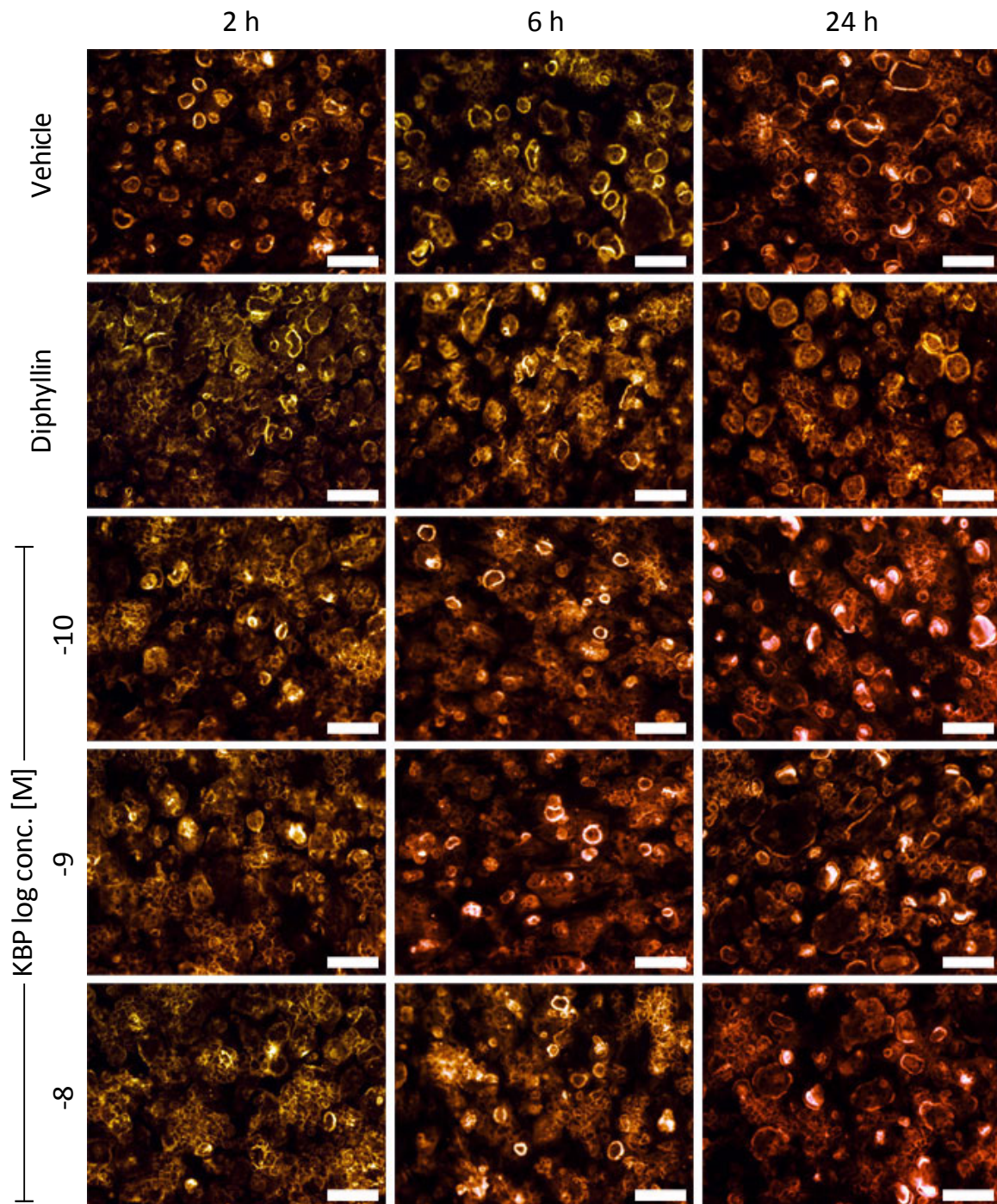


Figure 15. Actin rings in osteoclasts treated with KBP or diphyllin at 2, 6 or 24 hours. Mature human osteoclasts were seeded on slices of bovine cortical bone (50,000 cells per well), left to attach for 24 h and cultured in the presence of either KBP (0.1, 1 or 10 nM), diphyllin (200 nM) or vehicle (medium) as well as M-CSF and RANKL for 2, 6 and 24 h, respectively. Cells were fixed with formaldehyde and stained for actin using phalloidin-TRITC. Scale bars correspond to 200 μ m.

7.2.2 Actin staining of KBP (0.0001 – 1 nM)

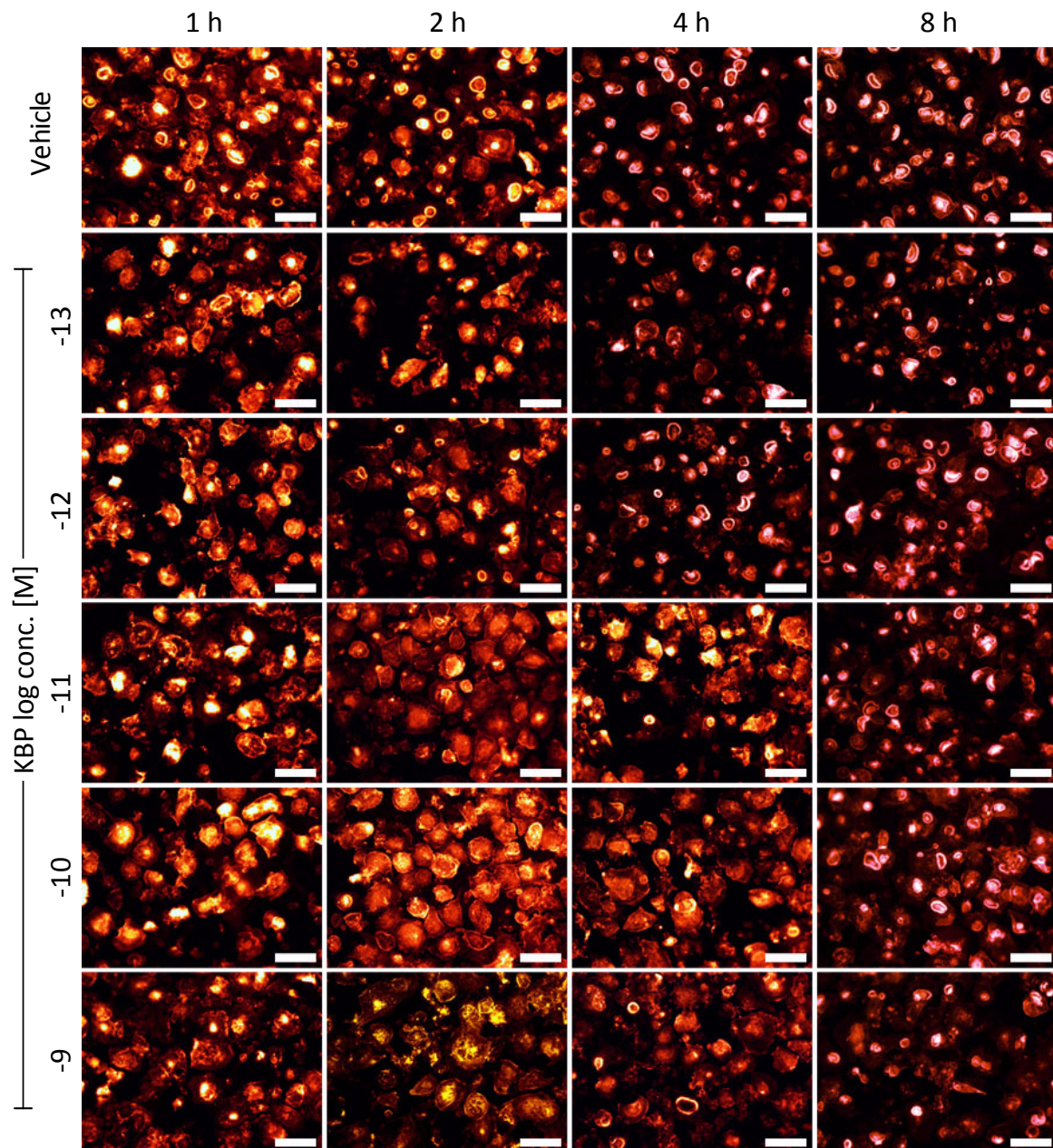


Figure 16. Actin rings in osteoclasts treated with KBP at 1, 2, 4 or 8 hours. Mature human osteoclasts were seeded on slices of bovine cortical bone (30,000 cells per well), left to attach for 24 h and cultured in the presence of either KBP (0.0001, 0.001, 0.01, 0.1, 1 or 10 nM) or vehicle (medium) as well as M-CSF and RANKL for 1, 2, 4 and 8 h, respectively. Cells were fixed with formaldehyde and stained for actin using phalloidin-TRITC. Scale bars correspond to 200 μ m.

7.2.3 Actin staining of KBP vs. sCT (0.00001 – 10 nM)

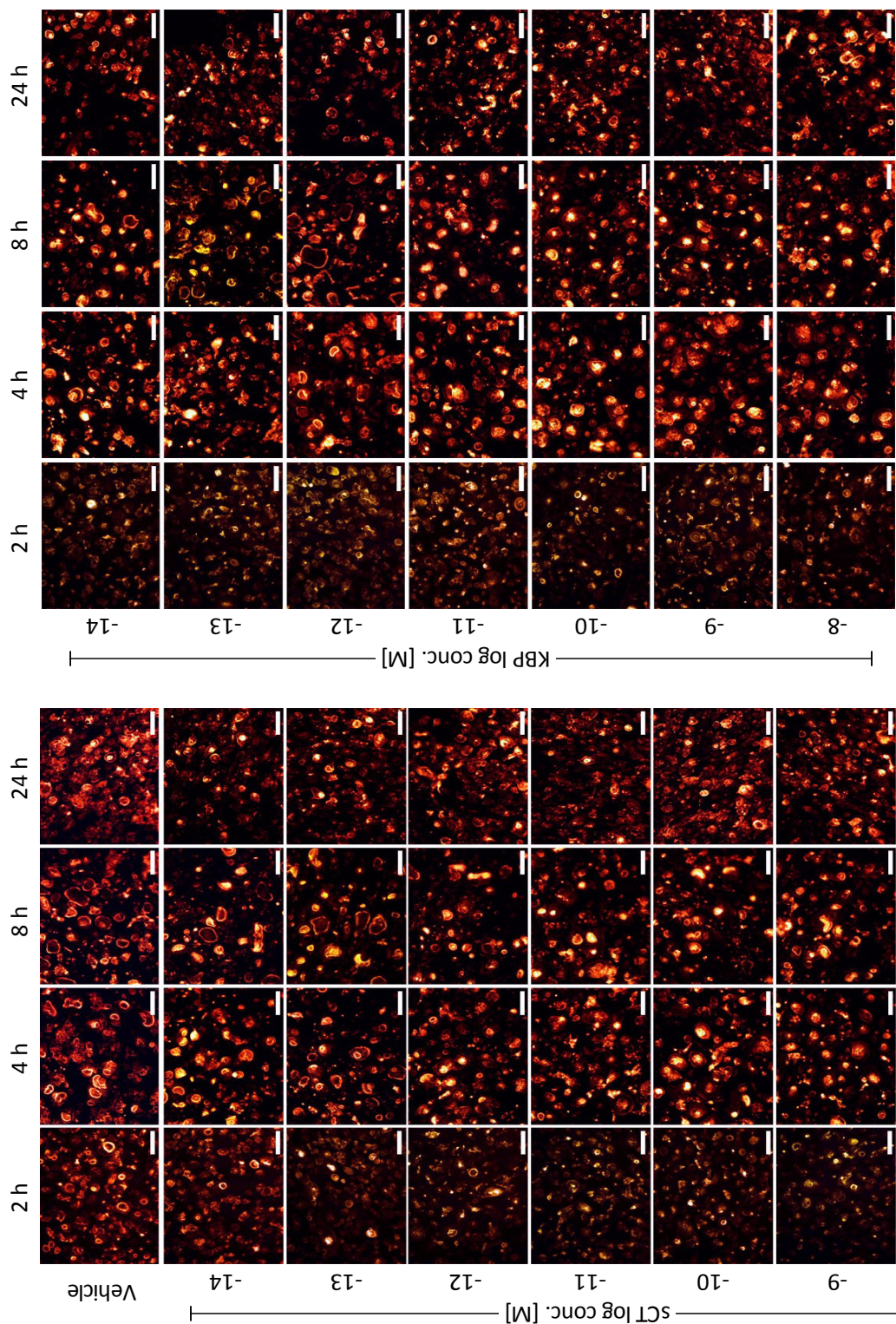


Figure 17. Actin rings in osteoclasts treated with KBP or sCT for 2, 4, 8 or 24 hours. Mature human osteoclasts were seeded on slices of bovine cortical bone (30,000 cells per well, 6 biological replicates), left to attach for 48 h and cultured in the presence of either KBP (0.00001 – 10 nM), sCT (0.00001 – 10 nM) or vehicle (medium) as well as M-CSF and RANKL for 2, 4, 8 and 24 h, respectively. Cells were fixed with formaldehyde and stained for actin using phalloidin-TRITC. Scale bars correspond to 200 µm.

8. Abstract

Osteoarthritis is the most common arthritic condition primarily affecting the elderly. Pathological alterations manifest in all tissues of the joint. The loss of cartilage followed by a narrowing of the joint space is the most obvious morphological symptom. However, in recent years, alterations of the periarticular bone received increasing attention, as they might promote disease progression. Due to the osteoclast's unique ability to resorb bone, researchers started to investigate its role in osteoarthritis.

In my thesis, I intended to shed light on the role of osteoclasts in osteoarthritis *in vitro* and *in vivo*. More precisely, I attempted to answer the following questions:

1. How do cartilaginous joint tissues alter the phenotype of osteoclasts *in vitro*?
2. Can the sCT analog KBP inhibit osteoclastic bone resorption and possibly outperform its natural equivalent *in vitro*?
3. How does the impairment of bone resorption affect subchondral bone and articular cartilage structure in a mouse model of osteoarthritis?

To answer these questions, I cultured mature human osteoclasts on bovine cortical bone, subchondral bone and articular cartilage and treated them with inhibitors targeting key processes of bone resorption. Subsequently, I measured parameters of bone and cartilage degradation as well as protein expression. Further, I treated mature human osteoclasts cultured on bovine cortical bone with KBP or sCT and evaluated their resorptive behavior. Lastly, I examined cartilage degradation in knee joint sections of osteoarthritic mice lacking the ability to form osteoclasts or resorb bone.

I found that osteoclast phenotypes *in vitro* are dependent on the tissue they reside on. Osteoclasts are capable of cartilage resorption as indicated by markers for collagen type II breakdown. This activity appears to be mediated by MMPs. However, cartilaginous matrices also shorten the cells life span. Further, I found that KBP inhibits bone resorption of osteoclasts *in vitro* with an effect subsiding after 24 hours. Kinetically, however, it was not superior over sCT. A lack of time prevented me from a comprehensive study of the osteoarthritis mouse model. A loss of cartilage in a control mouse was found, but, unexpectedly occurred in the unoperated knee. Examination of more specimens is necessary to validate and characterize the model.

As a conclusion, my experiments indicate a role for osteoclasts on cartilaginous matrices. In osteoarthritis they might, thus, be involved in the structural changes of the subchondral bone. The particular role for MMPs could provide a possibility for pharmaceutical interventions, but the identification of the exact MMP remains a task for future projects. Although KBP could not outperform sCT in the inhibition of cortical bone resorption *in vitro*, further studies are necessary to determine whether this holds true in other systems. Moreover, previously demonstrated benefits of sCT for cartilage remain to be investigated for KBP.

9. Zusammenfassung

Osteoarthritis ist die häufigste Gelenkkrankheit und tritt vorwiegend im Alter auf. Die pathologischen Veränderungen betreffen das gesamte Gelenk. Das deutlichste strukturelle Merkmal der Krankheit, die Verringerung des Gelenkzwischenraumes, wird durch Knorpelverlust verursacht. In den letzten Jahren rückte jedoch der gelenknahe Knochen in den Fokus der Wissenschaft, da Veränderungen dort möglicherweise den Krankheitsverlauf beschleunigen. Die einzigartige Fähigkeit der Osteoklasten Knochen abzubauen führte zur genaueren Erforschung der Rolle dieses Zelltyps in Osteoarthritis.

In meiner Arbeit widmete ich mich der Untersuchung der Rolle der Osteoklasten in Osteoarthritis. Im speziellen wollte ich die folgenden drei Fragen klären:

1. In welcher Weise beeinflussen Knorpelgewebe des Gelenks den Phänotyp von Osteoklasten *in vitro*?
2. Kann das sCT Analogon KBP osteoklastischen Knochenabbau *in vitro* verhindern und dabei möglicherweise sein natürliches Vorbild übertreffen?
3. Wie wirkt sich das Unterbinden osteoklastischen Knochenabbaus in einem Osteoarthritis Mausmodell auf die Struktur von subchondralem Knochen und Gelenkknorpel aus?

Zur Klärung dieser Fragen kultivierte ich ausgereifte menschliche Osteoklasten auf kortikalem Knochen, subchondralem Knochen und Gelenkknorpel von Rindern. Ich verwendete Inhibitoren um Schlüsselmechanismen der Knochenresorption auszuschalten. Im Anschluss bestimmte ich Parameter von Knochen- und Knorpelabbau sowie die Proteinexpression. Außerdem behandelte ich ausgereifte menschliche Osteoklasten die ich auf kortikalem Rinderknochen kultivierte mit KBP oder sCT und bestimmte deren Verhalten im Hinblick auf Knochenresorption. Zuletzt untersuchte ich den Knorpelabbau in Kniegelenkquerschnitten osteoarthritischer Mäuse, denen entweder die Fähigkeit fehlt Knochen abzubauen oder Osteoklasten zu bilden.

Ich fand heraus, dass der Phänotyp von Osteoklasten *in vitro* gewebeabhängig ist. Sie können außerdem Knorpel abbauen wie ich anhand von Markern für den Abbau von Kollagen Typ II feststellte. Dieses Verhalten beruht vermutlich auf MMPs. Knorpelhaltige Substrate verringern jedoch auch die Lebensspanne der Zellen. Darüber hinaus fand ich, dass KBP Knochenresorption durch Osteoklasten *in vitro* inhibiert, der Effekt aber nach 24 Stunden abklingt. Kinetisch unterscheidet sich KBP jedoch nicht von sCT. Aus Zeitgründen gelang mir keine umfangreichere Untersuchung des Mausmodells. Die Kontrollmaus die ich untersuchte zeigte Knorpelverlust, entgegen meiner Erwartung jedoch im nicht operierten Knie. Die Auswertung weiterer Mäuse ist notwendig zur Validierung und Charakterisierung des Modells.

Zusammenfassend fand ich Hinweise darauf, dass Osteoklasten eine Rolle auf Knorpelgewebe spielen. Sie könnten daher an den strukturellen Veränderungen des subchondralen Knochens in Osteoarthritis beteiligt sein. Die spezielle Rolle der MMPs macht diese zu möglichen Zielen zukünftiger Medikamente. Um welche MMP es sich handelt muss in nachfolgenden Studien geklärt werden. Obwohl KBP in der Hemmung kortikalen Knochenabbaus *in vitro* sCT nicht überlegen zu sein scheint, sind Folgestudien nötig um zu klären ob sich dies auch in anderen Systemen widerspiegelt. Die bereits bekannten knorpelbegünstigenden Effekte von sCT müssten ebenfalls noch für KBP bestätigt werden.

Lebenslauf

Persönliche Daten

Name

Heiko Müller

Universitäre Ausbildung

10/2012– 06/2015

MSc Molekulare Biologie

Universität Wien, Wien, Österreich

Vertiefungsrichtung: Zellbiologie

Abschlussarbeit: „The Role of Osteoclasts in Osteoarthritis: Tissue-Dependent Phenotypes and Inhibition”

09/2008 – 02/2012

BSc Biologische Chemie

Hochschule Mannheim, Mannheim, Deutschland

Abschlussarbeit: „Gene Regulatory Effects of an Allatostatin B Homolog in *Platynereis dumerilii*”

Praktische Erfahrung

08/2014 – 04/2015

Masterarbeit

Nordic Bioscience A/S, Herlev, Dänemark

Die Rolle von Osteoklasten in Osteoarthritis

01/2014 – 03/2014

Praktikum

Medizinische Universität Wien, Institut für Krebsforschung, Wien, Österreich

Die Rolle des FGF Signalwegs in Darmkrebs

02/2013

Praktikum

Forschungsinstitut für Molekulare Pathologie, Wien, Österreich

Mechanismen der Ausbildung von Zellpolarität in der embryonalen Entwicklung von *Caenorhabditis elegans*

06/2012 – 08/2012

Praktikum

BASF SE, Limburgerhof, Deutschland

Pathogenität von *Rhizoctonia solani* gegenüber Mais

11/2011 – 01/2012

Bachelorarbeit

Max-Planck-Institut für Entwicklungsbiologie, Tübingen, Deutschland

Genregulatorische Effekte von Neuropeptiden in der Metamor-phose von *Platynereis dumerilii*

09/2010 – 02/2011

Praxissemester

Karlsruher Institut für Technologie, Karlsruhe, Deutschland

Identifikation von Proteinen die an der Ausbildung epithelialer Zellpolarität in *Oryzias latipes* beteiligt sind