Elina Kylmäoja

OSTEOCLASTOGENESIS FROM BONE MARROW AND PERIPHERAL BLOOD MONOCYTES

THE ROLE OF GAP JUNCTIONAL COMMUNICATION AND MESENCHYMAL STROMAL CELLS IN THE DIFFERENTIATION

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Abstract
Osteoclasts are multinuclear bone degrading cells differentiated from monocytes which can be isolated from bone marrow and peripheral blood. Complex signaling between osteoclast precursors and other bone cells, such as mesenchymal stromal cells (MSC) occurs during the differentiation. Gap junctional communication (GJC) is one of the mechanisms in the cell fusion. GJC can be modulated with several substances such as the specific GJC stimulators, antiarrhythmic peptides (AAP). Due to their promising clinical value in the treatment of cardiac disorders, the effects of AAPs in cardiac tissue are studied extensively. This study was conducted in order to investigate the roles of GJC and AAPs in bone cell cultures. Further, the contribution of the MSCs on the effects of AAPs was studied along with comparison of two types of osteoclastogenesis cultures with differing quantities of MSCs.

GJC in osteoclastogenesis was studied with both GJC inhibitors and stimulators in mouse monocyte line RAW 264.7 cells and primary cultures with bone marrow hematopoietic cells. The following studies were made with human monocytes from peripheral blood and bone marrow where the effects of AAP10 were investigated in normal and acidic environments. In addition, comparison of osteoclastogenesis from bone marrow and peripheral blood monocytes was carried out in in vitro cell cultures on bovine or human bone slices. The cells were analyzed with regard to multinuclearity, bone resorption and the expression of several osteoclast markers.

The results show that GJC is utilized in osteoclastogenesis, but it is not indispensable. GJC in monocytes can be stimulated with the AAPs during osteoclastogenesis, but the effects depend on the culture conditions as well as on the presence of MSCs in the culture. The AAPs can also activate the MSCs leading to indirect regulation of osteoclastogenesis, as the MSCs produce several molecules affecting the differentiation. Further, monocytes from peripheral blood showed increased potential for osteoclastogenic differentiation compared to bone marrow derived monocytes. This can be explained by the presence of the osteoclastogenesis-controlling MSCs in the bone marrow culture, while the peripheral blood cultures contain only few of these cells and thus lack their regulatory effects.

Keywords: antiarrhythmic peptide, bone marrow, bone resorption, connexin, gap junction, monocyte, osteoclast, peripheral blood
Tiivistelmä


Asiastat: antiarytrmin peptidi, aukkoliitos, konneksiini, luun resorptio, luuydin, monosyytti, osteoklasti, perifeerinen veri
"A wise man once told me that mystery is the most essential ingredient of life, for the following reason: mystery creates wonder, which leads to curiosity, which in turns provides the ground for our desire to understand who and what we truly are."

- The Archivist in *The Secret History of Twin Peaks* by Mark Frost

*To cells, for their ability to divide enabling this study, and above all, for creating the most important things in my life, my children*
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Elina Kylmäoja
Abbreviations

18GA 18α-glycyrrhetinic acid
AAP Antiarrhythmic peptide
ALP Alkaline phosphatase
ATCC American type culture collection
BM Bone marrow
BMP Bone morphogenetic protein
BMU Bone multicellular unit
BRC Bone remodeling compartment
BSA Bovine serum albumin
C3a Complement factor 3a
cAMP Cyclic adenosine monophosphate
CD Cluster of differentiation
Cfba1 Core binding factor alpha 1
CFU-GM Colony forming unit for granulocytes and macrophages
CFU-M Colony forming unit for macrophages
CIC-7 Chloride channel 7
CR Calcitonin receptor
CT-1 Cardiotrophin-1
CTHRC1 Collagen triple-helix repeat-containing 1
Cx Connexin
DAB Diaminobenzidine
DC-STAMP Dendritic cell-specific transmembrane protein
D-MEM Dulbecco’s Modified Eagle Medium
DMP1 Dentine matrix protein 1
DMSO Dimethyl sulfoxide
ECM Extracellular matrix
FBGC Foreign body giant cell
FBS Fetal bovine serum
FCγRIII FCγ receptor III
FESEM Field emission scanning electron microscopy
FGF Fibroblast growth factor
FITC Fluorescein isothiocyanate
FRAP Fluorescence recovery after photobleaching
FSD Functional secretory domain
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
\textit{Gja1} \hspace{1em} \text{Gap junction protein alpha 1 gene}

GJC \hspace{1em} \text{Gap junctional communication}

HCl \hspace{1em} \text{Hydrochloric acid}

HLA-DR \hspace{1em} \text{Human leukocyte antigen – antigen D-related}

HSC \hspace{1em} \text{Hematopoietic stem cell}

ICTP \hspace{1em} \text{C-terminal telopeptide of type I collagen}

IFN \hspace{1em} \text{Interferon}

IP\textsubscript{3} \hspace{1em} \text{Inositol trisphosphate}

IL \hspace{1em} \text{Interleukin}

LPS \hspace{1em} \text{Lipopolysaccharide}

mAb \hspace{1em} \text{Monoclonal antibody}

M-CSF \hspace{1em} \text{Macrophage colony-stimulating factor}

M-CSFR \hspace{1em} \text{Macrophage colony-stimulating factor receptor}

MEPE \hspace{1em} \text{Matrix extracellular phosphoglycoprotein}

MMP \hspace{1em} \text{Matrix metalloproteinase}

mRNA \hspace{1em} \text{Messenger RNA}

MSC \hspace{1em} \text{Mesenchymal stromal cell}

NFATc1 \hspace{1em} \text{Nuclear factor of activated T-cells 1}

NF-\kappa B \hspace{1em} \text{Nuclear factor kappa-B}

OC-STAMP \hspace{1em} \text{Osteoclast stimulatory transmembrane protein}

ODDD \hspace{1em} \text{Oculodentodigital dysplasia}

OPG \hspace{1em} \text{Osteoprotegerin}

OSCAR \hspace{1em} \text{Osteoclast-associated receptor}

Osx \hspace{1em} \text{Osteoblast-specific transcription factor Osterix}

PB \hspace{1em} \text{Peripheral blood}

PBS \hspace{1em} \text{Phosphate buffered saline}

PFA \hspace{1em} \text{Paraformaldehyde}

PKC \hspace{1em} \text{Protein kinase C}

PPR \hspace{1em} \text{Pattern recognition receptor}

PTH \hspace{1em} \text{Parathyroid hormone}

QOP \hspace{1em} \text{Quiescent osteoclast precursor}

qPCR \hspace{1em} \text{Quantitative polymerase chain reaction}

RANK \hspace{1em} \text{Receptor activator of nuclear factor kappa-B}

RANKL \hspace{1em} \text{Receptor activator of nuclear factor kappa-B ligand}

RGD \hspace{1em} \text{Amino acid motif Arg-Gly-Asp}

RM \hspace{1em} \text{Osteoclastogenesis protocol with RANKL and M-CSF}
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>RMTD</td>
<td>Osteoclastogenesis protocol with RANKL, M-CSF, dexamethasone and TGF-β</td>
</tr>
<tr>
<td>Ror2</td>
<td>Tyrosine kinase-like orphan receptor protein 2</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SIRPα</td>
<td>Signal-regulatory protein alpha</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-box binding protein</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRACP</td>
<td>Tartrate resistant acid phosphatase</td>
</tr>
<tr>
<td>TRAF-6</td>
<td>Tumor necrosis factor receptor associated factor 6</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine-isothiocyanate</td>
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<tr>
<td>V-ATPase</td>
<td>Vacuolar ATP-ase</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
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<tr>
<td>Wnt</td>
<td>Wnt (Wingless-related integration site) signaling pathway</td>
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<tr>
<td>α-MEM</td>
<td>Minimum essential medium, alpha modification</td>
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List of original publications

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1 Introduction

Bone as a concept might be easily associated with death, fossils and remnants of previous life as well as to a fixed supporting scaffold for other organs of the body. However, a look through a microscope reveals a lively and actively working community of cells residing within the inorganic minerals of the bone. The cells are responsible for the lifelong turnover and maintenance of the bone tissue, remodeling, which leads to a complete renewal of the human skeleton decennially.

In addition to the functions within the bone tissue, the studies during the last few decades have shown that the bone cells have several other functions extending throughout the whole body. For instance, the bone and immune system cells act together in many immunological processes, a field of science which gained even its own term in the beginning of the 21st century, osteoimmunology. Furthermore, as more and more evidence of bone cell functions outside the bone tissue have started to emerge, bone has even been suggested to be a controller organ of the whole body similar to the brain.

The cells responsible for bone degradation during remodeling are osteoclasts, multinuclear cells differentiated from monocytic precursors through several cell fusion processes. Albeit osteoclastogenesis has been well studied over the years, the differentiation phases and the details of the osteoclast precursor cells are still not fully understood. Gap junctional communication (GJC) is one of the mechanisms in the cell fusion during the differentiation, but also its roles in the complex signaling network in the bone tissue are not fully characterized. The specific GJC stimulators, antiarrhythmic peptides (AAP) have shown promising effects on cardiac cells and the treatment of cardiac symptoms, but their effects in bone tissue are studied to a lesser degree. Therefore, the aim of this study was to investigate the role of GJC in osteoclastogenesis as well as the effects of the AAPs on the differentiating monocytes. Moreover, considering the complex regulatory pathways and the signaling between the bone cells, the role of the mesenchymal stromal cells (MSC) during osteoclastogenesis was studied. A better understanding of the functions of bone cells within the bone tissue as well as in the whole body will facilitate the development of new treatments for bone disorders such as osteoporosis and inflammatory bone loss, the prevalence of which are rapidly increasing due to the aging population of the world.
2 Review of the literature

2.1 Bone

Bones are often understood as rigid structures existing solely for supporting and protecting the soft tissues in the body. However, a deeper look reveals that bone is an active tissue having many vital functions such as maintenance of calcium and phosphate homeostasis, regulation of pH level and production of hematopoietic cells in the bone marrow. In addition, bone is constantly renewed in a process called remodeling where old or weak bone is replaced with newly formed bone tissue. Remodeling occurs throughout life enabling the repair of bone microdamage and maintenance of the skeletal homeostasis. Since remodeling is a tightly controlled phenomenon, errors in it can lead to several bone diseases such as osteoporosis (increased bone degradation) or osteopetrosis (increased bone formation) (Katsimbri, 2017). Another important phenomenon occurring in bone called modeling is characterized as bone shaping or reshaping without the locally coupled bone degradation and formation. Modeling activity appears during development and growth, but continues also in adult bone tissue (Hattner, Epker, & Frost, 1965; Langdahl, Ferrari, & Dempster, 2016).

The adult human skeleton consists of over two hundred bones which can be anatomically classified as flat bones, such as skull bones, and long bones, such as humerus and femur. Morphologically, bone tissue, a specialized form of dense connective tissue, can be classified as compact or cancellous bone. Of the total bone mass, 80% is compact bone, whereas the remaining 20% consists of cancellous bone. Since cancellous bone has a high surface to volume ratio, it represents about 80% of the total bone surface (Hadjidakis & Androulakis, 2006). Compact or cortical bone is a dense matter found in the shaft or diaphysis of the long bones where it forms a hollow cylinder protecting the inner bone marrow cavity. Cancellous or spongy bone consists of a lattice of bone trabeculae surrounded by bone marrow. Cancellous bone is present in the ends of long bones, epiphyses, which are covered with a thin layer of compact bone. The metaphysis, located between the diaphysis and the epiphysis, contains the cartilaginous growth plate responsible for bone growth during childhood. As skeletal maturity is reached, the cartilage in the growth plate is replaced with bone in a process called growth plate closure (Deng & Liu, 2005). The outer surface in most bones is a connective tissue sheath called the periosteum which contains collagen fibers, blood vessels and
osteogenic cells. The inner surface lining the bone marrow cavity, the endosteum, covers also the trabeculae in cancellous bone as well as the vascular Haversian and Volkmann’s canals of the bone. Endosteum consists of connective tissue fibers, stromal cells and the osteoblast lineage bone lining cells, which cover the inactive bone surfaces not undergoing remodeling (Dierkes et al., 2009; Kierszenbaum & Tres, 2016). The anatomical structure of bone is illustrated in figure 1.

Besides the anatomical and morphological classification, bone can be divided into lamellar and woven bone based on the disposition of the collagen fibers. In woven bone, which is present in the developing bone and during fracture repair, the arrangement of the collagen fibers is disorderly. This causes woven bone to be weak, and therefore, after formation it is subsequently replaced with lamellar bone. The stronger lamellar bone characterized by the ordered arrangement of the collagen fibers is present in the mature skeleton. In lamellar compact bone the mineralized bone matrix appears as thin layers or lamellae, which are concentrically aligned around a Haversian canal, a blood vessel channel running longitudinally within the bone tissue. These units are called osteons or Haversian systems, and they are separated from the adjacent interstitial lamellae with a thin cement line (Kierszenbaum & Tres, 2016). The cement lines are collagen deficient structures remaining from the preceding reversal phase of remodeling where bone resorption is switched to bone formation. Cancellous bone also has lamellar disposition, but instead of being arranged around the Haversian canal in an osteon, the lamellae in cancellous bone are organized parallel to the trabecular surface. Cancellous bone surfaces can contain hemiosteons, structures resembling half osteons, which represent previous remodeling activity and are separated from the underlying trabecular tissue with a similar cement line as seen in osteons. The hemiosteons lack Haversian canals, since they are in direct contact with the bone marrow cavity and thus receive nutrients directly from it (Burr & Allen, 2014).
Fig. 1. The anatomical structure of bone. Long bones can be divided into epiphysis, metaphysis and diaphysis. Bone marrow cavity resides inside the bone shaft in the diaphysis region. Diaphysis and epiphysis surfaces consist of compact bone, whereas epiphysis and metaphysis contain cancellous bone. The microanatomical structure of the compact bone consists of osteons with a central Haversian canal. Cancellous bone is a spongy-like tissue with bone trabeculae surrounded by bone marrow. The outer bone surfaces are covered with periosteum, whereas the inner surface lining the marrow cavity and covering the trabeculae is called endosteum. Modified from Kierszenbaum et al., 2016.

2.2 Bone matrix

In addition to cells, bone tissue contains extracellular matrix (ECM), which constitutes the major part of the bone tissue. Bone ECM can be divided into inorganic (65%) and organic (20-25%) components, while the remaining 10% is water. The inorganic phase consists of calcium phosphate in the form of hydroxyapatite crystals. The organic phase is mostly type I collagen (90%), but also small amounts of type III and V collagen exist around bone cells. The remaining 10% of the organic phase constitutes noncollagenous proteins such as osteopontin, osteocalcin and osteonectin, which have various activities during embryogenesis, but also control mineralization and participate in signaling and attachment of cells in the mature bone tissue. The orderly arrangement of the collagen fibrils and the hydroxyapatite crystals within and between the fibrils make bone hard and strong.
but also a resilient composite material (Burr & Allen, 2014; Kierszenbaum & Tres, 2016).

2.3 Bone cells

Bone contains several types of cells of mesenchymal and hematopoietic origin, which together enable bone growth and allow the bones to respond to changes in mechanical loading while maintaining the body’s mineral balance. The bone resorbing multinuclear cells, osteoclasts, are responsible for the initial bone degradation in the remodeling process, after which osteoblasts are recruited to the remodeling site to create new bone. As new bone tissue is formed, some osteoblasts become embedded inside the bone tissue and differentiate into osteocytes, which have an ability to sense mechanical changes in the bone tissue and further control the activity of osteoclasts and osteoblasts. Bone lining cells cover quiescent bone surfaces not undergoing remodeling (Florencio-Silva, Rodrigues da Silva Sasso, Sasso-Cerri, Simões, & Cerri, 2015). Osteal macrophages are bone tissue macrophages, which participate in bone modeling, remodeling and fracture healing (Cho, 2015). The bone cells communicate with each other through complex signaling pathways extending even to other organs, which reflects the essential role of the bone tissue in the homeostasis of the whole body (Florencio-Silva et al., 2015).

2.3.1 Osteoclasts

Osteoclasts are the only cells capable of resorbing the mineralized bone tissue. They are differentiated from monocyte/macrophage lineage cells by fusion from their mononuclear precursors (Quinn, Sabokbar, & Athanasou, 1996). Human osteoclasts are large, up to 300 µm in diameter, and have approximately five to eight nuclei (Bar-Shavit, 2007; Väänänen & Laitala-Leinonen, 2008), although in Paget’s bone disease osteoclasts with as much as 100 nuclei have been observed (Roodman & Windle, 2005). Osteoclasts are highly specialized for the bone degradation process, and recently they have also been found to have several other functions in maintaining the bone homeostasis, such as regulation of hematopoiesis, bone angiogenesis and bone formation. In the beginning of the 2000s, a completely new branch of bone science, osteoimmunology, was introduced when osteoclasts and the immune system were found to have a tight relationship (Arron & Choi, 2000).
Osteoclastogenesis

The multinuclear osteoclasts are generated from mononuclear hematopoietic monocyte/macrophage lineage cells by several cell fusion processes. The main osteoclast precursor cell has been shown to be a monocyte lineage cell expressing cluster of differentiation (CD) molecule CD14, but lacking CD16 expression (Costa-Rodrigues, Fernandes, & Fernandes, 2011; Hemingway et al., 2011; Komano, Nanki, Hayashida, Taniguchi, & Miyasaka, 2006; Massey & Flanagan, 1999; Nicholson et al., 2000). This CD14+CD16- monocyte is called the classical monocyte (K. L. Wong et al., 2011). CDs are different types of cell surface molecules, which can act for instance as receptors or ligands. CD14 is a pattern recognition receptor (PRR) for bacterial lipopolysaccharide (LPS), the major component of the bacterial cell wall (Zanoni & Granucci, 2013). CD16, also known as Fcγ receptor III (FcγRIII), is a receptor for IgG antibodies. It is expressed in natural killer cells, monocytes and neutrophils, and regulates their functions in response to inflammatory signaling (Moldovan et al., 1999).

Osteoclast precursors are present in bone marrow as well as circulating in peripheral blood (Matsuzaki et al., 1998; Shalhoub et al., 2000; Udagawa et al., 1990). Mizoguchi et al. presented the theory of the so-called cell cycle-arrested quiescent osteoclast precursors (QOP), which are differentiated from the earlier stage hematopoietic precursors (Mizoguchi et al., 2009). Due to the arrested cell cycle they are unable to replicate and are therefore committed to differentiate into osteoclasts. Muto et al. (Muto et al., 2011) showed that in mice, the QOPs detected in peripheral blood migrated to bone morphogenetic protein (BMP) disks implanted into dorsal muscle pouches. The disks induced the formation of ectopic bone and contained mature osteoclasts which were shown to have differentiated from the circulating QOPs. The authors suggested a theory that the osteoclast precursors initially differentiate into QOPs in hematopoietic organs such as bone marrow or spleen, subsequently enter the peripheral blood for transient circulation, and finally migrate to bone tissue for the final differentiation into mature osteoclasts (Fig. 2). This theory is supported by an early study of Walker (Walker, 1975) as well as a later study by Kotani et al. (Kotani et al., 2013), who showed that osteoclast precursors migrated from one mouse to another through surgically created shared blood circulation, parabiosis, and differentiated into mature osteoclasts in the bones of the second mouse (Kotani et al., 2013).
Fig. 2. The route of circulating quiescent osteoclast precursors (QOP) from hematopoietic organs to bone via blood vessels. After the initial differentiation from progenitor cells in hematopoietic organs, the QOPs enter the circulation. The final differentiation and fusion into multinuclear osteoclasts occurs after homing onto bone surfaces. Modified from Muto et al., 2011.

Osteoclastogenesis proceeds through complex signaling and tight control regulated by several growth factors, receptors and transcription factors. The multipotent hematopoietic stem cell (HSC) in bone marrow can differentiate into lymphocytes, erythrocytes, thrombocytes, granulocytes and mononuclear phagocytes. The HSC differentiates first into a colony-forming unit for granulocytes and macrophages (CFU-GM) in response to secretion of macrophage colony-stimulating factor (M-CSF) from the osteoblast lineage cells, while the expression of the M-CSF receptor (M-CSFR) and the transcription factor PU.1 begin to be expressed in the HSC (Bar-Shavit, 2007). The differentiation proceeds to CFU for macrophages (CFU-M), after which the expression of receptor activator of nuclear factor kappa-B ligand (RANKL), receptor activator of nuclear factor kappa-B (RANK), tumor necrosis factor receptor associated factor 6 (TRAF-6), nuclear factor κB (NF-κB) and c-Fos differentiate the CFU-M cells into preosteoclasts (Asagiri & Takayanagi, 2007). Another important transcription factor acting downstream to RANKL and NF-κB signaling is nuclear factor of activated T-cells 1 (NFATc1), which induces the expression of several genes typical of osteoclasts such as tartrate resistant acid phosphatase (TRACP), cathepsin K and calcitonin receptor (CR) (J. H. Kim & Kim, 2014). The stages of the differentiation are illustrated in figure 3.
The preosteoclasts next fuse into multinuclear cells. The fusion process is mediated by several factors, such as syncytin-1 (Søe et al., 2011), CD47/signal-regulatory protein alpha (SIRPα) complex (Lundberg et al., 2007), dendritic cell-specific transmembrane protein (DC-STAMP) (Yagi et al., 2005), osteoclast stimulatory transmembrane protein (OC-STAMP) (Yang et al., 2008) and gap junctions (Ilvesaro, Väänänen, & Tuukkanen, 2000; Schilling et al., 2008). The fusion process is not random since different fusion factors were shown to function at different time points during the fusion, suggesting that the fusion occurs only between certain types of preosteoclasts at a certain stage (Hobolt-Pedersen, Delaissé, & Søe, 2014; Møller, Delaissé, & Søe, 2017). In the final phase of osteoclastogenesis the multinuclear osteoclast differentiates into a bone resorbing osteoclast as the genes controlling the adherence and resorptive activity, such as αβ3 integrins and cathepsin K are expressed (Boyle, Simonet, & Lacey, 2003).

**Fig. 3.** The stages of differentiation in osteoclastogenesis. A hematopoietic stem cell (HSC) differentiates into multinuclear osteoclast through several phases (A), which are regulated by different factors, such as receptor activator of nuclear factor kappa-B ligand (RANKL), macrophage colony-stimulating factor (M-CSF) and osteoprotegerin (OPG) secreted from the osteoblast lineage cells (B). Modified from Bar-Shavit, 2007.
The two essential osteoclastogenic factors, M-CSF and RANKL, are produced by the stromal or osteoblast lineage cells. The importance of these molecules to osteoclastogenesis was noted already in the 1980s, when osteoclasts were generated in vitro in cocultures of bone marrow cells and osteoblasts (Takahashi et al., 1988b). The signaling and reciprocal regulation of osteoclast and osteoblast lineages is today well known, and includes three main components, namely RANK, RANKL and osteoprotegerin (OPG). RANK is a TNF receptor superfamily transmembrane protein expressed in osteoclasts, which binds its ligand, RANKL (Nakagawa et al., 1998). RANKL, also a TNF-family molecule, can exist as a membrane-bound form or it can be cleaved to yield a soluble form (Lacey et al., 1998). The membrane-bound form was recently shown to be active during the skeletal growth in mice while the soluble form is required for the normal bone remodeling in adult mice (J. Xiong et al., 2018). Osteoblast produced OPG is a decoy receptor for RANKL, and by binding to it, OPG can inhibit the binding of RANKL to RANK thus preventing osteoclastogenesis (Simonet et al., 1997; Tsuda et al., 1997; Yasuda et al., 1998). Thus, by regulating RANKL/OPG production, osteoblasts can control osteoclastogenesis (Yasuda et al., 1998).

**Bone resorption by osteoclasts**

Bone resorption sites form on microdamaged bone where osteocyte apoptosis and signaling from the surviving osteocytes recruit and activate osteoclasts (Cardoso et al., 2009; Kennedy et al., 2012; Kurata, Heino, Higaki, & Väänänen, 2006; Noble et al., 2003). Before the bone resorption process can begin, an osteoclast has to adhere to the bone surface. The structure attaching the osteoclast to bone is called the sealing zone. At first, the αvβ3 integrin present on the osteoclast plasma membrane recognizes the amino acid motif Arg-Gly-Asp (RGD) present in bone proteins such as osteopontin and bone sialoprotein and facilitates osteoclast attachment to the bone surface (Ross et al., 1993). Next, foot-like actin-rich processes, podosomes, accumulate in clusters on the osteoclast’s bone facing surface mediating the primary attachment. After new actin deposition and reorganization of the actin filaments a sealing zone is formed, thus creating an isolated microenvironment for the bone resorption below the osteoclast (Bar-Shavit, 2007; Luxenburg et al., 2007).

In addition to the sealing zone three other membrane domains exist in the resorbing osteoclast, namely ruffled border, basolateral membrane and functional secretory domain (FSD), which is located in the center of the basolateral membrane.
The ruffled border acts as the osteoclast’s bone resorbing organelle, whereas the FSD serves as a region for disposal of the bone degradation products (Salo, Metsikkö, Palokangas, Lehenkari, & Väänänen, 1996; Salo, Lehenkari, Mulari, Metsikkö, & Väänänen, 1997; Väänänen & Laitala-Leinonen, 2008).

Bone resorption constitutes both dissolution of the inorganic hydroxyapatite crystals as well as proteolytic cleavage of the organic compounds and takes place in a closed compartment, the Howship lacuna, below the ruffled border (Cappariello, Maurizi, Veeriah, & Teti, 2014). The dissolution of the inorganic matrix begins when acidic intracellular vesicles fuse to the plasma membrane below the osteoclast forming the ruffled border. The ruffled border secretes protons (H+) and chloride ions (Cl−) that combine to form hydrochloric acid (HCl), which acidifies the lacuna (Baron, Neff, Louvard, & Courtoy, 1985; Väänänen & Laitala-Leinonen, 2008). Protons are secreted with vacuolar ATP-ase (V-ATPase), a proton pump (Väänänen et al., 1990), and chloride ions with chloride channel 7 (ClC-7) (Kornak et al., 2001; Schlesinger, Blair, Teitelbaum, & Edwards, 1997).

Acidification and dissolution of the mineralized matrix are pivotal to the subsequent degradation of the organic matrix, and can occur several hours before the organic matrix resorption begins (Blair, Kahn, Crouch, Jeffrey, & Teitelbaum, 1986). The alkalization of the cytosol resulting from the proton secretion is resolved with a chloride/bicarbonate exchanger located in the basolateral membrane (Teti et al., 1989).

After demineralization, the organic constituents of the bone matrix become exposed and degraded by proteolytic enzymes such as matrix metalloproteinases (MMP) and cathepsin K (Everts et al., 2006). MMPs are a group of extracellular matrix degrading enzymes, and of these, MMP-9 has been shown to conduct the initial steps in bone organic matrix resorption by releasing the cross-linked carboxyterminal telopeptide of type I collagen (ICTP) (Parikka et al., 2001).

Cathepsin K is activated in low pH and responsible for the type I collagen, osteopontin and osteonectin degradation (Bossard et al., 1996; Votta et al., 1997). The importance of cathepsin K in bone remodeling has been demonstrated in several studies. Overexpression of cathepsin K in mice has been shown to increase osteoclastic bone resorption leading to decreased trabecular bone volume. Concurrently, overexpression leads to increased osteoblast numbers and enhanced bone formation, which together with increased resorption causes a high bone turnover rate (Kiviranta et al., 2001; Morko et al., 2009). In cathepsin K-deficient mice the disturbed osteoclastic bone resorption is compensated with an osteoblast-produced increased RANKL/OPG ratio supporting osteoclastogenesis. Although
Osteoclast numbers are high in these mice, the overall bone turnover rate is decreased resulting in increased bone formation, osteopetrosis (Kiviranta et al., 2005). Further, osteoclast-specific cathepsin K deletion in mouse hematopoietic cells led to decreased bone resorption but increased osteoclast and osteoblast numbers as well as enhanced bone formation (Lotinun et al., 2013). This effect is mediated by the increased RANKL/OPG ratio as well as increased production of sphingosine-1-phosphate (S1P) in osteoclasts, which stimulates osteoblast differentiation, survival and migration (Lotinun et al., 2013; Pederson, Ruan, Westendorf, Khosla, & Oursler, 2008; Ryu et al., 2006).

Cathepsin K activates also another bone degrading enzyme, TRACP (Ljusberg et al., 2005). After the early studies recognizing the two forms of TRACP, isoforms 5a and 5b (Lam, Eastlund, Li, & Yam, 1978), it was later shown that TRACP is synthesized as a single polypeptide chain in the form of a loop (5a) and later activated by the enzymatic cleavage of the loop-peptide by cathepsin K leading into a disulfide-linked two-subunit form (5b) (Ljusberg, Ek-Rylander, & Andersson, 1999). TRACP is expressed intracellularly in osteoclasts, macrophages and dendritic cells, but the main isoform secreted from macrophages and dendritic cells is TRACP 5a (Janckila et al., 2002; Janckila et al., 2005), whereas TRACP 5b is secreted from osteoclasts (Halleen et al., 2000). After cathepsin K has been secreted to the resorption lacuna and it has started to degrade the organic matrix, the bone degradation products as well as cathepsin K are thereafter endocytosed into the osteoclast (Salo et al., 1997). The endocytic vesicles fuse with TRACP containing vesicles where cathepsin K cleaves TRACP into TRACP 5b (Vääräniemi et al., 2004; Halleen, Tiitinen, Ylipahkala, Fagerlund, & Väänänen, 2006). TRACP 5b continues the organic matrix degradation inside the transcytotic vesicles by producing reactive oxygen species (ROS), which assist in collagen fragmentation (Halleen et al., 1999). TRACP 5b level in serum correlates with osteoclast number and is used as a clinical marker of bone resorption activity (Halleen et al., 2006). The transcytotic vesicles containing the bone resorption products are finally targeted to the FSD and secreted into extracellular fluid. The structure of the bone-resorbing osteoclast is illustrated in figure 4.
The roles of osteoclasts in hematopoiesis and osteoimmunology

In addition to bone resorption, osteoclasts have a few other tasks in the bone tissue. They have been shown to participate in regulation of hematopoiesis with the release of cathepsin K and MMP9, which can control the maintenance and mobilization of HSCs from the HSC niche in the bone marrow (Kollet et al., 2006). Osteoclasts can also regulate angiogenesis within the bone tissue by releasing factors such as vascular endothelial growth factor (VEGF) (Trebec-Reynolds, Voronov, Heersche, & Manolson, 2010) and heparanase (Saijo et al., 2003) which stimulate endothelial cells to form blood vessels. Osteoclasts contribute to angiogenesis also indirectly.
by controlling osteoblasts, which also participate in the process (Liu et al., 2016). The bone formation control activity of osteoclasts during remodeling is discussed in detail in chapter 2.6.

Although it was found already in the 1970s that certain immune cells can produce bone resorption activating molecules (Horton, Raisz, Simmons, Oppenheim, & Mergenhagen, 1972), the term osteoimmunology was not described until in 2000 by Arron & Choi after the findings that bone cells and the immune system can control each other (Arron & Choi, 2000). After almost two decades it has become clear that the immune and bone cells originate from the same precursors in the bone marrow, that complex signaling occurs between them, and that same cytokines affect their function. The major cells in this interplay are osteoclasts and T-cells. Inflammatory activation of T-cells can induce them to produce various osteoclastogenic or anti-osteoclastogenic cytokines, such as interleukins (IL), transforming growth factor-β (TGF-β), tumor necrosis factor (TNF) and interferons (IFN). The osteoclastogenic pro-inflammatory cytokines increase the expression level of RANKL, the main factor in osteoclast differentiation. The following increase in osteoclastogenesis and bone resorption is the basis behind the pathological bone loss in bone diseases involving inflammatory factors such as osteoporosis and rheumatoid arthritis (Zupan, Jeras, & Marc, 2013). In addition to the fact that T-cells can regulate osteoclasts, a few recent studies have shown that osteoclasts can reciprocally participate in immune responses by activating regulatory T-cells (Kiesel, Buchwald, & Aurora, 2009; H. Li et al., 2010).

Another key molecule regulating the osteoimmunological events is an IgG-like receptor in osteoclasts called the osteoclast-associated receptor (OSCAR). It is known to act as a costimulatory agent in osteoclastogenesis and activates also monocyte/macrophage lineage cells, albeit OSCAR is an orphan receptor without any known ligand (Nemeth et al., 2011). Mutations of the OSCAR gene have been linked to low bone mass and to increased risk for osteoporotic fractures (G. S. Kim et al., 2005), and increased expression of OSCAR has been reported in rheumatoid arthritis patients (Herman et al., 2008).

### 2.3.2 Osteoblasts

The multipotent mesenchymal stem cell pool, which gives rise to several cell types including fibroblasts, chondrocytes and adipocytes, can also produce the bone forming osteoblasts. In scientific literature the term mesenchymal stem cell has
long been abbreviated to MSC, but recently problems related to the nature and naming of these cells have been brought out. The nomenclature of these and the related stromal cells are discussed in chapter 2.4.2, and in this chapter the mesenchymal stem cell refers to the actual osteoblast stem cell. Osteoblast differentiation is regulated by several transcription factors of which Runt-related transcription factor 2 (Runx2), also known as core binding factor alpha 1 (cbfa1), and osteoblast-specific transcription factor, Osterix (Osx), are the most important (Capulli, Paone, & Rucci, 2014). Runx2 controls the expression of several osteoblast-specific genes and is critical for osteoblastogenesis, since in Runx2-deficient mice bone tissue ossification is completely prevented due to maturational arrest of osteoblasts (Komori et al., 1997). Other important signaling pathways involved in osteoblastogenesis are Wingless-related integration site (Wnt) and BMP pathways (Capulli et al., 2014). Several WNT proteins can activate different cell surface receptors and their signaling is divided into canonical (or WNT \( \beta \)-catenin) and non-canonical pathways. The canonical WNT signaling in osteoblasts induces osteoblastogenesis and bone formation. However, both pathways are important for maintenance of bone homeostasis, since the different WNTs can either activate or inhibit both osteoblasts and osteoclasts (Lerner & Ohlsson, 2015). Similarly, various BMPs are involved in osteoblast differentiation and bone formation, some BMPs inducing these processes and some acting as inhibitors. Both BMPs and WNTs can cooperate with TGF-\( \beta \) in complex signaling pathways regulating the functions of osteoblasts (G. Chen, Deng, & Li, 2012).

After the initial differentiation from mesenchymal stem cells to osteoblast progenitor cells, the cells undergo a phase of proliferation where the cells start to express bone matrix proteins such as type I collagen and fibronectin. These pre-osteoblasts develop further into cuboidal cells and begin the expression of alkaline phosphatase (ALP) (Capulli et al., 2014). ALP is responsible for the degradation of inorganic pyrophosphate, a calcification inhibitor, into organic phosphate, which is required for the bone mineralization (Millán, 2013). The proliferation phase proceeds to the final phase of extra-cellular matrix synthesis after matrix proteins accumulate in the cells. The differentiated osteoblasts secrete first the organic matrix components resulting in the formation of a non-mineralized matrix called osteoid. Finally, osteoblasts mineralize the bone matrix by secreting vesicles containing hydroxyapatite crystals, which are then arranged between the collagen fibrils (Capulli et al., 2014; Neve, Corrado, & Cantatore, 2011).

After matrix synthesis is completed, osteoblasts either undergo apoptosis or differentiate into osteocytes or bone lining cells. However, similar to osteoclasts,
also osteoblasts have recently been noted to be involved in various other functions. They have a crucial role in controlling osteoclastogenesis by secretion of the osteoclastogenic molecules M-CSF, RANKL and OPG (Bar-Shavit, 2007; Lacey et al., 1998; Yasuda et al., 1998). Other suggested roles for osteoblasts include control of the hematopoietic stem cell niche (Calvi et al., 2003) and endocrine cell function through production of osteocalcin (Karsenty & Ferron, 2012).

2.3.3 Osteocytes

Osteocytes represent the final differentiation phase of osteoblasts when the cells have become trapped within the newly formed bone matrix. During the embedment osteoblasts start to differentiate into osteocytes and undergo various morphological changes, which transform the cuboidal osteoblasts into stellate shape osteocytes with numerous (40-100) long cell processes extending from the cell body. During differentiation the cell volume and number of organelles in osteoblasts decrease significantly. Expression of several regulatory genes is crucial for the differentiation and as a consequence the cells start to express proteins such as dentine matrix protein 1 (DMP1), matrix extracellular phosphoglycoprotein (MEPE), fibroblast growth factor 23 (FGF23) and sclerostin. These proteins are essential for the osteocyte’s functions in controlling mineral and phosphate homeostasis and bone remodeling (H. Chen, Senda, & Kubo, 2015). For instance, sclerostin, encoded by the SOST gene, is an endogenous inhibitor of Wnt signaling and therefore decreases bone formation by osteoblasts. Monoclonal antibodies against sclerostin have proved to be effective drugs for the treatment of osteoporosis since they represent the few anabolic agents leading to increased bone formation (Shah, Shoback, & Lewiecki, 2015).

Over 90% of bone cells are osteocytes residing within the bone matrix in small spaces called lacunae. The lacunae are connected to each other with narrow canals called canaliculi, where the osteocyte cell processes run comprising a complex network called the lacunar-canalicular system inside the bone. This network contains circulating fluid that supplies nutrients and oxygen for osteocytes (H. Chen et al., 2015).

The osteocyte processes are connected to each other as well as to osteoblasts, bone lining cells, endothelial cells and hematopoietic cells in the endosteal surfaces with gap junctions (H. Chen et al., 2015). Gap junctions are small channels between cells enabling the exchange of small molecules from one cell to another, and they are discussed in detail in chapter 2.8. The osteocyte signaling network allows them
to accomplish their functions in bone tissue, which are mechanosensing and signal transportation. Osteocytes can translate mechanical stimuli such as bone loading or microfractures into biochemical signals and subsequently regulate the actions of other bone cells (Bonewald, 2007). Osteocytes are the main regulators in bone remodeling, since they can control both osteoblasts and osteoclasts (Capulli et al., 2014). The essential role of osteocytes in remodeling has been demonstrated for instance in studies showing that osteocyte apoptosis resulting from bone microdamage increases osteoclast numbers and subsequent resorption in the area (Cardoso et al., 2009; Noble et al., 2003). The apoptotic osteocytes have been shown to secrete increased levels of RANKL and M-CSF (Kurata et al., 2006) and to stimulate the surrounding non-apoptotic osteocytes to produce increased levels of RANKL thereby promoting osteoclast differentiation (Kennedy et al., 2012).

The discovery of the functions of other bone cells outside the bone tissue seems to hold true also for osteocytes, which can be considered as endocrine cells based on their ability to secrete FGF23. FGF23 is a hormone which transports signals from bone to kidney, where it controls phosphate and vitamin D metabolism (A. Martin et al., 2011). Moreover, osteocytes were revealed to have a considerable role in regulation of lymphopoiesis and fat metabolism, as Sato et al. showed that mice lacking osteocytes suffer from drastic lymphopenia and are totally exclusive of white adipose tissue (Sato et al., 2013). Based on their results, the authors suggest an astonishing idea that bone tissue could perhaps control the functions of other organs similar to the way brain acts as the major controller organ in the body.

2.3.4 Bone lining cells

Bone lining cells are characterized as flat cells covering the quiescent bone surfaces not undergoing bone remodeling. They have been shown to have various functions in bone tissue, although their precise origin and properties are still not fully understood. After bone matrix synthesis, osteoblasts can transform into bone lining cells, which have been shown to be connected to each other and to osteocytes within the bone matrix with gap junctions. The gap junctional coupling enables the transport of small molecules between the bone tissue and bone marrow, while the layer of bone lining cells serves as a biological membrane between the two above-mentioned tissues (Burr & Allen, 2014; Dierkes et al., 2009).

Although the lining cells cover the resting bone surfaces, they are not dormant cells. Everts et al. (Everts et al., 2002) have shown that the lining cells are able to remove and digest the nonmineralized collagen fibrils protruding from the bone
surface with MMPs before osteoclastic bone resorption. A similar activity of the lining cell can be seen after an osteoclast has made a resorption pit, as lining cells are able to clean the resorption pits from the collagen remnants left over by the osteoclast. Finally, the lining cells form the cement line and deposit a thin layer of collagen along the pit, after which osteoblasts are able to attach to the pit for formation of the new bone. Supporting the evidence for the matrix degradation activity of the lining cells, Dierkes et al. (Dierkes et al., 2009) showed that lining cells express several types of MMPs as well as their physiological tissue inhibitors (TIMPs), which take part in the matrix degradation process. Moreover, by analyzing cbfa-1 gene expression, they conclude that the lining cells are an intermediate cell type between osteoblasts and osteocytes. However, the proof for the lining cells being the actual reversal cells preparing the bone surface for bone formation after resorption is still lacking, and also other cells such as osteal macrophages have been proposed to serve this function.

The relation of bone lining cells to osteoblasts is interesting, especially as it turned out that administration of parathyroid hormone (PTH) can transform the quiescent lining cells into active osteoblasts (S. W. Kim et al., 2012). Secondly, Kristensen et al. suggested that osteoblasts could be recruited from bone lining cells during bone remodeling (Kristensen, Andersen, Marcussen, Rolighed, & Delaisse, 2014). Furthermore, Matic et al. (Matic et al., 2016) showed that the lining cells could in fact be the major source of osteoblasts in adult bone tissue in contrast to the mesenchymal stem cells, which are the osteoblast precursors during development.

2.3.5 Osteal macrophages

Most tissues contain a population of resident macrophages, some of which have been specifically named, such as Kupffer cells in liver, Langerhans cells in skin and microglia in brain. In 1984, Hume et al. (Hume, Loutit, & Gordon, 1984) described the presence of stellate shaped F4/80-positive macrophages in both endosteal and periosteal surfaces of bone. These bone tissue macrophages were named osteal macrophages or osteomacs by Chang et al. (Chang et al., 2008), as they described the presence of these cells among bone lining cells in endosteal and periosteal bone surfaces as well as in the bone lining tissue in trabecular bone. Their study showed that the presence of osteomacs significantly increased bone mineralization by osteoblasts and that osteomacs formed a canopy-like structure over bone modeling sites, indicating that osteomacs can regulate osteoblast function during bone
formation. Moreover, the study revealed the crucial role of osteomacs in bone modeling, as depletion of osteomacs led to a complete loss of the osteoblast bone-forming surface. Since their naming, osteomacs have been found to have various roles in maintaining the bone homeostasis.

In addition to their essential role in bone modeling, osteomacs have also been suggested to participate in bone remodeling. F4/80-positive macrophages were shown to be present near osteoclasts at remodeling sites (Alexander et al., 2011; Batoon, Millard, Raggatt, & Pettit, 2017; Wu, Raggatt, Alexander, & Pettit, 2013), although it is currently not known how osteomacs affect the remodeling events. It has been proposed (Batoon et al., 2017) that osteomacs might act as phagocytes removing apoptotic osteoblasts or preventing the leaking of the remodeling mediator molecules into the bone marrow space. They could also be the cells controlling the reversal phase where bone resorption is shifted to bone formation. As described earlier, bone lining cells have also been thought to serve this function. However, in a recent review article concerning the functions of osteomacs Batoon et al. (Batoon et al., 2017) suggested that the reversal cells could actually be osteomacs, since earlier studies (Tran Van, Vignery, & Baron, 1982) had shown that the reversal phase is mediated by mononuclear phagocytic cells. Also in another review article Miron and Bosshardt (Miron & Bosshardt, 2016) suggested that osteomacs might control the reversal phase by secreting TGF-β and ephrin B2, which seem to be the potential coupling factors between osteoclasts and osteoblasts.

Related to their functions in modeling and remodeling, osteomacs play a significant role in bone repair after fracture. They have been shown to be present during callus formation (Hankemeier et al., 2001) and to promote intramembranous bone healing (Alexander et al., 2011). In their study, Alexander et al. showed that depletion of osteomacs led to diminished bone formation, and, furthermore, that the administration of M-CSF increased osteomac numbers at the injury site and therefore augmented new bone formation and mineralization.

### 2.4 Bone marrow

Bone marrow is the soft, spongy tissue inside the hollow bone cylinder lined by cortical bone extending within the trabeculae of cancellous bone at the end of long bones. Bone marrow can be classified as red marrow, which consists of hematopoietic tissue, or yellow marrow, which consist mainly of adipocytes (fat cells). Most bones in children contain the red marrow, which is responsible for production of blood cells. In adults the red marrow is present only in a few flat
bones, such as skull, sternum, scapulae, vertebrae, ribs, pelvic bones and at the ends of long bones, such as femur and humerus (Porwit, McCullough, & Erber, 2011). In other adult bones the red marrow is replaced with yellow marrow, which acts as an energy source and probably regulates lipid metabolism (Burr & Allen, 2014).

Like all other tissues, bone marrow consists of the parenchyma (the functional part of the tissue) and the stroma (the supporting framework of the tissue). Bone marrow parenchyma contains the hematopoietic cells including both precursor and mature blood cells (Porwit et al., 2011). The stroma of the bone marrow contains macrophages, fibroblasts, blood vessels and intercellular matrix, as well as a complex and heterogeneous population of the so-called stromal cells. These include mesenchymal stem cells, reticular cells and adipocytes (Anthony & Link, 2014).

2.4.1 Hematopoietic stem cells and hemopoiesis

All blood cells are derived from a common multipotent hematopoietic stem cell (HSC) present in the bone marrow. HSCs proliferate and differentiate into mature blood cells through several stages. The first differentiation step generates the lineage committed progenitors, common myeloid and lymphoid progenitors (Porwit et al., 2011). The myeloid progenitors continue differentiation towards the following intermediate cell types including CFU-GM and CFUs for eosinophils, basophils, erythrocytes and megakaryocytes. Next, these cells generate the several types of maturing blood cells such as monoblasts, myeloblasts, erythroblasts and megakaryoblasts. The lymphoid progenitors differentiate into T- and B-cell precursors. The final differentiation stages produce all mature blood cells: monocytes, granulocytes, erythrocytes, megakaryocytes, lymphocytes and plasma cells (Kierszenbaum & Tres, 2016). After maturation the blood cells and platelets enter blood vessels inside the bone marrow and migrate into the bloodstream (Porwit et al., 2011). As described in chapter 2.3.1, osteoclasts are derived from the differentiation of CFU-GM into CFU-M and further to preosteoclast, and the initial differentiation occurs in bone marrow (Bar-Shavit, 2007). CFU-GM cells can also differentiate into another multinuclear cell resembling an osteoclast, the foreign body giant cell (FBGC). FBGCs are observed in the granulation tissue which develops around implanted materials as a foreign body reaction. FBGCs have roles in inflammatory diseases and bone loss, and although they appear similar to osteoclasts, they do not have the ability to resorb bone (Sheikh, Brooks, Barzilay, Fine, & Glogauer, 2015; ten Harkel et al., 2015).
HSCs in bone marrow are located in a so-called niche, a microenvironment created by the surrounding cells that support and regulate the HSCs. The niches have been found near endosteum as well as close to arterioles and sinusoids, and they consist of the HSCs and the supportive stromal/osteoblast lineage cells. The HSC niche is regulated by several factors such as cytokines and growth factors along with Wnt and TGF-β signaling (Zhao & Li, 2015), and the main cells responsible for this are the stromal/osteoblast lineage cells (Anthony & Link, 2014; Calvi et al., 2003; J. Zhang et al., 2003). As previously mentioned, osteoclasts can also control the HSC niche (Kollet et al., 2006).

### 2.4.2 Mesenchymal stromal cells

Based on the early studies of the stromal cells (Friedenstein, Piatetzky-Shapiro, & Petrakova, 1966; Friedenstein, Chailakhyan, Latsinik, Panasyuk, & Keiliss-Borok, 1974), the term mesenchymal stem cell was introduced by Caplan in 1991. These cells were shortly thereafter isolated from various tissues and described to have a self-renewal capacity as well as the ability to differentiate into all mesenchyme-derived cells, such as osteoblasts, chondrocytes, adipocytes and fibroblasts (Caplan, 1991; Klimczak & Kozlowska, 2016). Although the term mesenchymal stem cell is still widely used, mostly because of the recent great interest into stem cell-based therapies for a variety of different disorders, a change to the nomenclature of these cells has been suggested (Caplan, 2017; Dominici et al., 2006; Horwitz et al., 2005). The suggestion is based on the fact that the stromal cell population in the bone marrow is very heterogenic, and most of these cells are not actually stem cells in the sense that they are not all multipotent and self-renewing (Bianco, Robey, & Simmons, 2008; Bianco, 2014; Horwitz et al., 2005; Ramakrishnan, Torok-Storb, & Pillai, 2013; Rasini et al., 2013). Instead, the stromal cell population consists of different types of cells expressing various surface molecules of which none are specific for the whole cell population (Rasini et al., 2013). The minimal criteria for defining the multipotent stromal cells has been proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy and includes the expression of cell surface molecules CD105, CD73 and CD90 without the expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and human leukocyte antigen – antigen D-related (HLA-DR). In addition, the cells must be plastic adherent and have the ability to differentiate into osteoblasts, adipocytes and chondroblasts (Dominici et al., 2006).
Interestingly, *in vivo*, the stromal cells were found to be located on the outer walls of the large bone marrow blood vessels, sinusoids. Therefore, they were considered as pericytes, the cells surrounding blood vessels throughout the body (Bianco, 2014; Crisan *et al.*, 2008). In fact, these mesenchymal/stromal cells have been isolated from the walls of many types of different sized blood vessels from several tissues (Nombela-Arrieta, Ritz, & Silberstein, 2011). Surprisingly, a recent study challenged the idea of the pericytes being the mesenchymal/stromal cells, since pericytes isolated from several tissues did not behave as stem cells for other cell types *in vivo* (Cano, Gebala, & Gerhardt, 2017; Guimarães-Camboa *et al.*, 2017). Therefore, the identity and biology of the MSC/pericyte remains to be solved. Based on all the aforementioned facts, it has been suggested that the abbreviation MSC should no longer refer to mesenchymal stem cells, but rather, for example, to marrow stromal cells, multipotent mesenchymal stromal cells or medicinal signaling cells (Bianco, 2014; Caplan, 2017; Dominici *et al.*, 2006; Horwitz *et al.*, 2005). In this study, these cells are hereafter referred to as mesenchymal stromal cells with an abbreviation MSC.

The main tasks of the MSCs are the regulation of bone homeostasis and regeneration as well as the maintenance of the hematopoietic stem cell niche (Bianco, 2014). In addition, they have many immunoregulatory effects (Ghannam, Bouffi, Djouad, Jorgensen, & Noël, 2010). *In vitro*, the MSCs appear as plastic-adherent fibroblast-shaped cells (Bianco *et al.*, 2008; Horwitz *et al.*, 2005). Although the MSCs are widely studied *in vitro*, it has been remarked that these cultures obviously do not represent the situation *in vivo*, as the cells seem to lose some of their characteristics during culture. Therefore, the details of the reciprocal regulation between the hematopoietic cells and MSCs *in vivo* are not yet understood in detail (H. Li, Ghazanfari, Zacharaki, Lim, & Scheding, 2016). However, the *in vitro* studies have proven that the MSCs have the ability to regulate osteoclastogenesis either by producing various osteoclastogenesis promoting factors (Haynesworth, Baber, & Caplan, 1996; Mbalaviele *et al.*, 1999) or inhibitory molecules such as OPG (Oshita *et al.*, 2011) and CD200 (Varin, Pontikoglou, Labat, Deschaseaux, & Sensebé, 2013). The reason behind these contradictory effects have been explained to be the ability of the MSCs to exert distinct effects on osteoclastogenesis depending on the inflammatory state of the tissue (Sharaf-Eldin, Abu-Shahba, Mahmoud, & El-Badri, 2016).

As described earlier, the quiescent bone surfaces are covered with the single layer of bone lining cells. The endosteal bone marrow side has been shown to contain another cell layer of epithelial-like mesenchymal stromal cells termed
marrow sac (Bi, Simmons, Hawkins, Cox, & Mainous, 2000). The marrow sac in human bone was later shown to be one or two cells thick and to contain cells expressing osteoblast markers (Bi, Mainous, Yngve, & Buford, 2008). The authors suggest that these cells could serve as an osteoprogenitor pool capable of differentiating into osteoblasts on demand.

### 2.5 Bone modeling

Bone modeling is defined as bone shaping or reshaping without the coupled activities of osteoclasts and osteoblasts at a certain site and can be categorized into formation modeling performed solely by osteoblasts, or resorptive modeling performed by osteoclasts. Occurring mostly during development and growth, the purpose of modeling is to reshape the bones allowing both longitudinal and radial growth and to adjust the bones to changing mechanical loads. In modeling both bone resorption and formation can occur, but they take place on different, pre-existing bone surfaces (Burr & Allen, 2014).

Modeling in adult bone was first described by Hattner, Epker and Frost in 1965, when they found differences in cement lines in several human bones (Hattner et al., 1965). They reported that while almost 97% of the cement lines present in cancellous bone had a scallop-like morphology, the remaining 3% were smooth. They presumed that the scalloped cement lines represent previous osteoclast and thus remodeling activity, whereas the smooth ones are evidence of bone formation without previous resorption, i.e. bone modeling. This finding was later supported by other studies showing that modeling occurs in both adult rat (Erben, 1996) and human bone (Kobayashi et al., 2003) throughout life. In fact, modeling has been shown to cause the increase in bone mass following the administration of some osteoporosis drugs. Lindsay et al. (Lindsay et al., 2006) showed that treatment with teriparatide, a PTH analogue, induces modeling-based bone formation in cancellous and endocortical bone surfaces. In addition, denosumab, an anti-RANKL-antibody also used to treat osteoporosis, has been shown to increase bone mineral density (Cummings et al., 2009) probably by allowing modeling-based bone formation while simultaneously inhibiting both remodeling and modeling-based bone resorption (Ominsky et al., 2015).

Modeling is composed of two kinds of activities: activation and subsequent bone resorption or formation. In the activation stage osteoclast or osteoblast precursor cells are recruited to the modeling site where they differentiate into mature cells and start their activities. These events are presumably similar to bone
remodeling and are discussed in more detail in chapter 2.6. As previously described, osteoclasts play a crucial role in modeling (Chang et al., 2008), as they create the protective canopy structure over the bone-forming modeling sites. The modeling process continues until the demands for the altered circumstances in the bone tissue environment have been met (Burr & Allen, 2014).

2.6 Bone remodeling

Bone remodeling, the replacement of old bone with newly formed bone tissue, is a process by which the bones are able to maintain their strength and mineral homeostasis. In contrast to modeling, where bone formation and resorption take place in different sites and perhaps at different times, in remodeling the activities of osteoblasts and osteoclasts are temporally and spatially coupled. This means that new bone is formed exactly in the same anatomical location where it was formerly resorbed, and is a way for bones to repair microfractures. Remodeling begins already in fetal bones and continues throughout life, being the main bone turnover process in adulthood and increasing with age (Katsimbri, 2017). The dynamic feature of the bone tissue is represented by the high number of remodeling units called basic multicellular units (BMU) present in the adult human skeleton. Approximately 3-4 million BMUs begin their activities in one year, and about 1 million BMUs are operating at any given time. The remodeling process in a BMU lasts 6-9 months, and roughly 10% of the skeleton is renewed within one year. As a consequence, the whole adult skeleton is renewed decennially (Manolagas, 2000). As 80% of the bone surface is cancellous bone, remodeling is more active in cancellous bone compared to cortical bone (Hadjidakis & Androulakis, 2006).

The remodeling process can be divided into five stages: activation, resorption, reversal, formation and termination. The activation signal can be mediated by mechanical force or soluble mediators, such as certain hormones, growth factors, cytokines and adhesion molecules, which regulate the differentiation of osteoblasts and osteoclasts (Katsimbri, 2017; Manolagas, 2000). As previously described, osteocyte apoptosis caused by bone microdamage induces RANKL secretion from adjacent osteocytes thereby promoting the activation and resorption stages in remodeling (Kennedy et al., 2012). Examples of the hormones regulating the activation of remodeling are PTH, vitamin D₃ and estrogen. PTH and the active form of vitamin D₃, 1α,25(OH)₂D₃ induce RANKL secretion from stromal cells and osteoblasts thus promoting osteoclastogenesis in vitro (Huang et al., 2004; Takahashi et al., 2014). In vivo, however, vitamin D₃ seems to have the opposite
effect as it suppresses bone resorption. Estrogen inhibits remodeling by preventing bone resorption and maintaining bone formation (Khosla, Oursler, & Monroe, 2012).

Remodeling of cancellous bone takes place in a so-called bone remodeling compartment (BRC), which was first described by Hauge et al. (Hauge, Qvesel, Eriksen, Mosekilde, & Melsen, 2001). They showed that the remodeling sites are covered by flat cells forming the so-called canopy structure above the bone surface (Fig. 5). They supposed that the canopy cells originate from the bone lining cells, since they expressed many osteoblastic cell markers, such as ALP, osteocalcin and osteonectin. According to their hypothesis, the lining cells detach from the bone surface and form a protective roof over the bone remodeling site. After remodeling is completed, new bone lining cells are formed from the osteoblasts present on the newly formed bone surface, and finally the old and new lining cell layers fuse into a single layer covering the bone. However, the origin of the canopy cells was later challenged by another study by Kristensen et al. (Kristensen et al., 2014), which suggested that the marrow sac, the other layer of cells above the lining cell layer on the bone marrow side, would actually be the layer creating the canopy. They proposed that when bone resorption begins, the marrow envelope is lifted and covers the remodeling site. In their hypothesis the bone lining cells covering the adjacent quiescent bone surface are in continuity with the flat canopy cells. In addition, Kristensen et al. suggested that osteoblasts could be recruited to the BRC either by differentiation from the marrow envelope forming the canopy or from the bone lining cells. The third possible route could be from the capillaries, which were noticed to be present near the BRCs (Andersen et al., 2009). In this study, osteoclast precursor cells were found in and near these capillaries suggesting that the function of the capillaries is to provide the osteoclast and possibly osteoblast precursors to the BRC. The purpose of the BRC is probably to act as a closed microenvironment enabling the exchange of essential molecules and cells between the bone tissue and extracellular fluid, and to serve as a site where bone resorption is linked to bone formation during the remodeling cycle (Hauge et al., 2001). The importance of the intact canopies in remodeling were shown in studies where damaged canopies correlated with problems in bone formation (Andersen et al., 2009; Andersen, Soe, Sondergaard, Plesner, & Delaisse, 2010; Jensen et al., 2012) and where absent canopies were linked to the bone loss observed in post-menopausal osteoporosis patients (Andersen et al., 2014).

As a consequence of the activation stage, osteoclast precursors are recruited to the BRC, where they differentiate into mature osteoclasts. During the subsequent
resorption stage osteoclasts resorb the bone by mechanisms described in chapter 2.3.1. In cancellous bone, remodeling takes place on the bone surface under the canopy. In cortical bone, remodeling proceeds in a tunnel through the bone tissue around the Haversian canals when osteoclasts resorb the bone in the so-called cutting zone. The blood vessel in the Haversian canal provides the precursor cells and other mediators in a similar way to the capillaries associated with the cancellous bone BRC (Sims & Martin, 2014).

After resorption is completed, the reversal phase follows and switches bone resorption to bone formation, a phenomenon referred to as coupling. The precise nature of the reversal cell preparing the bone surface for bone formation is still unknown, although the lining cells and osteomacs have been suggested to be these cells. Andersen et al. (Andersen et al., 2013) showed that the reversal surfaces are covered with flat cells expressing osteoblast markers such as Runx2 and ALP. These cells seem to differentiate into bone-forming osteoblasts during the reversal phase, since the cells closer to the osteoid surface were more cuboidal and expressed Osterix, which is a later marker of osteoblast differentiation. Raggatt and Partridge (Raggatt & Partridge, 2010) proposed a probable theory of both osteoblast lineage cells and osteomacs acting together during the reversal phase, with osteomacs being responsible for removing first the resorption remnants and the osteoblast lineage cells subsequently forming the cement line.

The osteoblast lineage reversal cell is also one candidate for producing the coupling signals which are required for the beginning of bone formation. A number of complex coupling signals have been proposed, such as matrix-derived molecules (e.g. TGF-β), secreted molecules and membrane-bound mediators such as ephrins and their receptors (Sims & Martin, 2014). One of the most important coupling mechanisms is the signaling between osteoblasts and osteoclasts. Osteoblasts control the differentiation and function of osteoclasts through RANKL/RANK/OPG and Wnt5a-tyrosine kinase-like orphan receptor protein 2 (Ror2) signaling (Boyce & Xing, 2008; Maeda et al., 2012). In addition, osteoclasts can mediate coupling by secreting the coupling factors called clastokines such as cardiotrophin-1 (CT-1), BMP-6, Wnt10b, collagen triple-helix repeat-containing 1 (CTHRC1), complement factor 3a (C3a), semaphorin 4D and S1P, which control osteoblastogenesis and osteoblast activity (Charles & Aliprantis, 2014; Negishi-Koga & Takayanagi, 2012; Pederson et al., 2008; Ryu et al., 2006; Sims & Martin, 2014).

As a consequence of the coupling signals, osteoblasts start to synthesize the new bone matrix, osteoid. The formation phase lasts about 4-6 months, after which
50-70% of the osteoblasts undergo apoptosis and the rest become either bone lining cells or osteocytes. During the termination phase the osteoid is mineralized, and finally the bone returns to the quiescent stage (Katsimbri, 2017). The schematical illustration of the bone remodeling cycle is presented in figure 5.
2.7 Osteoclastogenesis \textit{in vitro}

2.7.1 The history of osteoclastogenesis \textit{in vitro} studies

The pioneering osteoclast \textit{in vitro} experiments performed in the 1980s relied on isolation of mature osteoclasts directly from bone tissue. The isolation process was difficult due to low numbers of osteoclasts present in the hard bone tissue. In addition, these osteoclasts were already differentiated and often at the later stages of their life cycle, which limited the duration of the cell culture experiments (Hoebertz & Arnett, 2003). Around the same time, it was verified that the osteoclasts are differentiated from the hematopoietic precursors from the bone marrow or peripheral blood (T. J. Martin, 2013; Sabokbar & Athanasou, 2003; Walker, 1975). During the 1980s osteoclast cell culturing became easier, when it was noticed that osteoclasts could be generated from hematopoietic cells in cultures with osteoblast lineage cells (Chambers, 1982; Takahashi \textit{et al.}, 1988a; Takahashi \textit{et al.}, 1988b; Thomson, Mundy, & Chambers, 1987; Udagawa \textit{et al.}, 1990). Later on, the discovery of RANKL (Lacey \textit{et al.}, 1998; Yasuda \textit{et al.}, 1998), OPG (Simonet \textit{et al.}, 1997; Tsuda \textit{et al.}, 1997) and M-CSF (Tanaka \textit{et al.}, 1993) secretion from osteoblastic cells allowed the production of the recombinant forms of these molecules enabling the \textit{in vitro} generation of osteoclasts directly from HSCs. During the last years of the 1990s, it became clear that RANKL and M-CSF are sufficient for the differentiation of the HSCs into osteoclasts (Matsuzaki \textit{et al.}, 1998; Quinn, Elliott, Gillespie, & Martin, 1998). However, the addition of supplementary differentiation factors, such as TGF-\(\beta\) and the glucocorticoid dexamethasone have been reported to improve the differentiation potential of the precursors and to increase the bone resorption activity of the mature osteoclasts (Fuller, Lean, Bayley, Wani, & Chambers, 2000; Massey, Scopes, Horton, & Flanagan, 2001; Susa, Luong-Nguyen, Cappellen, Zamurovic, & Gamse, 2004). The osteoclastogenesis-stimulatory effect is shown to be mediated by TGF-\(\beta\), a co-stimulator of RANKL, which increases the survival of the precursors while inhibiting the osteoclastogenesis suppressor molecules (Fuller \textit{et al.}, 2000; Massey \textit{et al.}, 2001). Dexamethasone cooperates with TGF-\(\beta\) in stimulation of the precursor cells for differentiation simultaneously downregulating the expression of the inhibitory molecules such as IFN-\(\beta\) (Takuma \textit{et al.}, 2003). On the contrary, TGF-\(\beta\) has been reported to have conflicting effects on osteoclastogenesis and osteoclast activity, which are dependent on the presence of MSCs and the surrounding microenvironment (Houde, Chamoux, Bisson, & Roux, 2009; Karst, Gorny, Galvin, 48
& Oursler, 2004; Quinn et al., 2001). One of the mechanisms by which TGF-β inhibits osteoclastogenesis has been shown to be due to increased OPG production in stromal cells (Murakami et al., 1998; Takai et al., 1998). The opposing effects of TGF-β on osteoclastogenesis arise presumably from the various cell culture types as well as different concentrations used in these studies.

2.7.2 The rationale for the osteoclastogenesis in vitro studies

During the past 20 years of osteoclast in vitro cultures from HSCs, a large number of studies have been made to reveal the different mechanisms behind osteoclastogenesis, the signaling during this process, and the factors affecting it. Several drugs have been developed for the treatment of diseases associated with increased osteoclast activities and the inflammatory bone resorption, and for those, preclinical testing of candidate molecules with in vitro osteoclast culture models has been essential. Although the preliminary testing can be performed with animal cell cultures, it is well known that results obtained in animal cultures are not always applicable to human tissues. Therefore, the study of human osteoclast cultures is still needed. Another reason for this is that animal rights and ethics have become more important among the public as well as in the scientific community, which has led to efforts to reduce studies with laboratory animals.

The need for new therapeutic agents is still present, as the aging population is facing an increased number of age-related bone disorders. It is estimated that osteoporosis alone causes 9 million fractures globally per year (Johnell & Kanis, 2006). According to a report of the International Osteoporosis Foundation in 2013, the cost of osteoporotic fractures in the EU in 2010 was approximately 37 billion euros (Hernlund et al., 2013). The global cost of osteoporotic fractures is forecast to increase to 131.5 billion dollars by 2050 (Johnell, 1997). In addition to the high economical burden, the fractures cause harm and pain for the affected individuals and can lead to disability, loss of ability to work or even indirectly to death. The aforementioned facts besides the suboptimal treatments available, indicate that the need for research in osteoclast and bone biology is essential.

2.7.3 Osteoclast precursor sources for in vitro studies

Since HSCs are present in both bone marrow and peripheral blood, both tissues can be used as osteoclast precursor sources for in vitro experiments. Only a few studies have compared osteoclastogenesis from these different sources (Quinn, Neale,
Fujikawa, McGee, & Athanasou, 1998; Takahashi et al., 1989) and it is currently not known if there are differences between the osteoclasts generated from bone marrow or peripheral blood monocytes. The monocyte pool is known to be different in these tissues. In peripheral blood about 90% of the monocytes are the classical (CD14+CD16-) monocytes, whereas the other subsets, intermediate (CD14+CD16+) and non-classical (CD14-CD16+) monocytes together represent only about 10% of the population (K. L. Wong et al., 2011). The major type in bone marrow is the intermediate (CD14+CD16+) monocyte (Mandl, Schmitz, Weber, & Hristov, 2014).

All monocyte types have been shown to differentiate into osteoclasts in vitro (Sprangers, Schoenmaker, Cao, Everts, & de Vries, 2016). In their study, Sprangers et al. show that the different monocyte subsets respond differently to the pro-inflammatory cytokine IL-17A. They suggest that different types of osteoclasts could be formed from the different monocytes depending on the physiological or pathological conditions existing in the body. It is likely that the main osteoclast precursor during homeostasis is the classical monocyte, as shown previously (Costa-Rodrigues et al., 2011; Hemingway et al., 2011; Komano et al., 2006; Massey & Flanagan, 1999; Nicholson et al., 2000). During inflammation, the intermediate monocytes could possibly differentiate into osteoclasts with an increased bone resorption capacity, leading to the bone loss associated with many inflammatory conditions.

One of the differences between bone marrow and the peripheral blood cell population is the presence of MSCs. Both sources contain MSCs, but while they are relatively easy to isolate from bone marrow in sufficient numbers for cell culture (H. Li et al., 2016; Ramakrishnan et al., 2013), they are present in low numbers in peripheral blood (He, Wan, & Li, 2007; Kassis et al., 2006; Kuznetsov et al., 2001; Roufosse, Direkze, Otto, & Wright, 2004; Trivanović et al., 2013). The origin of the circulating MSCs is not known, but two sources have been suggested: bone marrow and the blood vessels containing pericytes (S. Wong et al., 2015). Both bone marrow MSCs and pericytes have been shown to migrate into injured tissues (Caplan, 2016; C. Chen, Corselli, Péault, & Huard, 2012; L. Li & Jiang, 2011). However, the true nature of the MSCs/pericytes circulating in physiological conditions as well as their relationship into each other is currently debated (Cano et al., 2017; Guimarães-Camboa et al., 2017). Either way, considering osteoclastogenesis studies in vitro, the greater MSC population in bone marrow samples and their osteoclastogenesis regulatory effects makes the choice of the monocyte source a highly important aspect to be considered.
2.8 Gap junctional communication

2.8.1 The structure and function of gap junctions

Cell fusion during osteoclastogenesis is enabled by the actions of several fusion proteins discussed in chapter 2.3.1. Another mechanism facilitating the fusion is gap junctional communication (GJC), which allows cells to intercellularly exchange small molecules, such as ions, metabolites and second messengers like cyclic adenosine monophosphate (cAMP), inositol trisphosphate (IP3) and calcium (Lo, 1999).

The gap junctions are formed when two proteins called connexons or hemichannels from adjacent cells dock into each other and form an aqueous channel. The undocked connexons/hemichannels can also be active and mediate signaling between the cell and the extracellular milieu. The connexons are comprised of six connexin (Cx) subunits arranged cylindrically around the central pore. The Cx subunit consists of four transmembrane domains, one intracellular and two extracellular loops and cytoplasmic N- and C-terminal domains (Yeager, Unger, & Falk, 1998). To date, 21 different connexin genes have been recognized in human and 20 in mouse (Nielsen et al., 2012). The different connexin proteins can form homo- or heteromeric connexons, and the pairing to form gap junctions can similarly occur in a homo- or heterotypic manner between identical or different connexons. The ability to modify the structure in such great versatility enables the formation of various types of channels with different functions, as gap junctions are present in most cells and have diverse roles in development, differentiation, proliferation and cell growth (Lo, 1999). On the cell surface, hundreds or even thousands of gap junctions assemble into gap junction plaques, which range in diameter from under 100 nm to several micrometers (Segretain & Falk, 2004).

The gap junction or hemichannel opening and closure called gating can be regulated by cytosolic pH, voltage, calcium ion levels and growth factors, which may affect the phosphorylation state of the connexins (Plotkin & Bellido, 2013). For instance, an intracellular increase in calcium level or acidification results in gap junction uncoupling (Nielsen et al., 2012).

In addition to forming the channels for molecule transport, an active role for the cytoplasmic C-terminal domain of some connexins without gap junction formation has been demonstrated. For instance, upon activation, the C-terminal end of Cx43 localizes to the nucleus and has been reported to have inhibitory actions on cell proliferation in several cell lines (Dang, Doble, & Kardami, 2003; Moorby et al., 2012).
& Patel, 2001; Olbina & Eckhart, 2003; Y. Zhang, Kaneda, & Morita, 2003). In addition, the Cx43 C-terminus can bind to several signaling complexes inside the cell and thus participate in various regulatory pathways (Hervé, Bourmeyster, Sarrouilhe, & Duffy, 2007).

2.8.2 Gap junctions in bone cells

Several studies have revealed the presence of gap junctions also in bone cells: between two adjacent osteoblasts or osteocytes, between osteoblasts and osteocytes (Civitelli et al., 1993; Doty, 1981; Jones et al., 1993; Yellowley, Li, Zhou, Jacobs, & Donahue, 2000), in osteoclasts (Ilvesaro et al., 2000; Schilling et al., 2008) as well as in the bone marrow stromal cells (Dorshkind, Green, Godwin, & Fletcher, 1993; Matemba, Lie, & Ransjö, 2006). The previously introduced osteocytes in the canalicular network that are connected to each other and also to osteoblasts and bone lining cells with gap junctions create together a syncytium, an interconnected web of cells allowing the fast transportation of signals within the bone tissue (H. Chen et al., 2015). The structure of gap junctions and hemichannels and the gap junctional network between the bone cells is illustrated in figure 6.

![Fig. 6. Gap junctions and hemichannels in bone cells. The bone tissue can be seen as a syncytium connected by gap junctions (a) and hemichannels (b) providing for rapid signaling within the tissue. Modified from Buo & Stains, 2014.](image-url)
The main connexin type expressed in bone is Cx43, along with the expression of Cx45, Cx40, Cx46 and Cx37 by the osteoblast lineage cells (Buo & Stains, 2014). In both humans and mice, mutations in the gap junction protein alpha 1 gene (Gja1), which encodes Cx43, results in oculodentodigital dysplasia (ODDD), a hereditary disease causing craniofacial, limb and neurological defects (Dobrowolski et al., 2008; Flenniken et al., 2005; Paznekas et al., 2003). The essential role of gap junctional signaling in bone development has been shown in mice lacking Cx43, which show delayed ossification and osteoblast dysfunction resulting in craniofacial and skeletal abnormalities (Lecanda et al., 2000). The severe malformations cause the mice to die shortly after birth. In addition, several studies of Cx43 deficiency have revealed that Cx43 is essential for the normal differentiation and function of osteogenic cells. The defects in osteoblast and osteocyte activities lead to problems in bone modeling, reduced bone mineral density and increased osteoblast and osteocyte apoptosis (Stains, Watkins, Grimston, Hebert, & Civitelli, 2014). Watkins et al. showed that ablation of the Cx43 gene Gja1 in the mouse osteogenic cell lineage led to lower secretion of the osteoclastogenesis inhibitor OPG from osteoblasts, therefore indirectly causing increased osteoclastogenesis (Watkins et al., 2011). Secondly, Zhang et al. described an indirect way to enhanced osteoclastogenesis after Cx43 ablation in mouse osteoblasts and osteocytes, where Cx43 deficient osteocytes had an increased RANKL/OPG messenger ribonucleic acid (mRNA) ratio supporting osteoclastogenesis (Y. Zhang et al., 2011).

2.8.3 Modulation of gap junctional communication in bone cells

Inhibition of GJC

GJC can be either inhibited or stimulated with certain agents. Gap junctions can be blocked with several compounds, for example heptanol and octanol (long carbon chain alcohols), glycyrrhetinic acid (compound from Glycyrrhiza glabra, better known as licorice root), carbenoxolone (semisynthetic derivative of glycyrrhetinic acid), quinine (alkaloid) and oleamide (fatty acid amide). In addition, GJC can be blocked with specific antibodies or small synthetic peptides having a similar amino acid sequence as a certain Cx molecule, thus called connexin-mimetic peptides (Manjarrez-Marmolejo & Franco-Pérez, 2016). The most frequently used Cx-mimetic peptides are Gap26 and Gap27, which contain a similar amino acid
sequence to the extracellular loops of Cx37, Cx40 and Cx43. The suggested mechanism of action is inhibiting the docking of the hemichannels by binding to the extracellular loops, thus preventing the formation of a functional gap junction (Evans & Leybaert, 2007).

The GJC blockers have been shown to have effects on osteoclast gap junctions and to cause problems in osteoclastogenesis. Ilvesaro et al. showed that heptanol and Gap27 inhibited the formation of multinuclear osteoclasts from rat mononuclear precursors and decreased both the total resorption area as well as the number of resorption pits (Ilvesaro et al., 2000; Ilvesaro, Tavi, & Tuukkanen, 2001). Ransjö et al. showed that 18α-glycyrrhetinic acid (18GA) and oleamide inhibited the PTH- and 1α,25-(OH)2D3-stimulated resorption pit formation of rat osteoclasts (Ransjö, Sahli, & Lie, 2003). Matemba et al. demonstrated the use of carbenoxolone for inhibition of osteoclastogenesis and the expression of osteoclast-specific genes (Matemba et al., 2006) and Schilling et al. (Schilling et al., 2008) showed that heptanol decreased the formation of multinuclear osteoclasts from human mononuclear precursors.

Stimulation of GJC

Specific stimulation of GJC can be induced with synthetic compounds known as antiarrhythmic peptides (AAPs). The first biological AAP was isolated from bovine heart tissue and found to improve the rhythmicity of cultured rat cardiomyocytes (Aonuma et al., 1980). Subsequently, several new AAPs were synthesized based on the amino acid sequence of the biological AAP, and of these, a peptide termed AAP10 proved to be the most potent. However, the low stability of the synthetic AAPs caused challenges in their use (Axelsen et al., 2007). In 2003, Kjølbye et al. developed a new improved and more stable AAP called rotigaptide, also known as ZP123. The structure of rotigaptide is very similar to AAP10, except that all L-amino acids are exchanged to D-amino acids, which inhibits the enzymatic degradation of the peptide (Kjølbye, Knudsen, Jepsen, Larsen, & Petersen, 2003).

The mechanism of action of AAPs in GJC stimulation has been suggested to be the protein kinase C (PKC)-mediated modulation of the Cx43 C-terminal end phosphorylation (Axelsen et al., 2006; Weng et al., 2002) and the increased Cx43 expression (Stahlhut, Petersen, Hennan, & Ramirez, 2006). Also other types of kinases have been reported to have GJC stimulatory effects, albeit with the problem that they do not act solely on connexins, but possibly also on complex signaling pathways having a number of other non-specific targets (Nielsen et al., 2012).
Several studies have reported that the AAPs are active only under metabolic stress, such as acidosis or hypoxia (Eloff, Gilat, Wan, & Rosenbaum, 2003; Haugan et al., 2005; Hennan et al., 2006; Jørgensen et al., 2005; Kjølbye et al., 2003; Wang, Zhang, Ruan, Liu, & Wang, 2007).

The GJC stimulatory and antiarrhythmic effects of AAPs in cardiac tissue have been demonstrated in several studies (Sun, Jiang, Zhao, & Hu, 2015; Clarke, Thomas, Petersen, Evans, & Martin, 2006; Dhein et al., 2010; Haugan et al., 2005; Hennan et al., 2006; Ni, Ruan, & Zhang, 2015). Recently, a promising effect of rotigaptide in reducing the myocardial infarction size in porcine heart and protecting human endothelial cell function after forearm venous occlusion was described by Pedersen et al. (Pedersen et al., 2016). In bone tissue the effects of AAPs are investigated only in one study, where rotigaptide was shown to increase GJC in human osteoblasts and to inhibit ovariectomy-induced bone loss in rats. Ovariectomy leads to estrogen deficiency causing decreased bone strength which in this study was prevented by rotigaptide, suggesting that it has effects on osteoblast and/or osteoclast activities (Jørgensen et al., 2005). The clinical relevance of GJC modulation could be to offer new options for developing treatments for bone diseases. If GJC could be cell-specifically inhibited in osteoclasts, it could lead to reduced osteoclastogenesis and alleviate diseases with increased bone resorption. On the contrary, GJC stimulation in osteoblasts could balance the disturbed remodeling towards bone formation, which has already been observed in rats (Jørgensen et al., 2005).
3 Aims of the study

Osteoclastogenesis as well as the detailed characteristics of the osteoclast precursor cells are currently not thoroughly studied. These issues are tightly linked to many bone diseases and age-related bone disorders. New therapeutic approaches and understanding of the signaling within the complex bone cell network are required.

The first hypothesis of this work was that GJC in bone tissue could be the therapeutic target in developing new treatments for bone disorders. GJC in osteoclast fusion has been only rarely investigated. A few studies have shown that inhibition of GJC leads to decreased osteoclastogenesis, but the effects of increased GJC in osteoclastogenesis have remained unknown. Since AAPs that enhance GJC have been shown to ameliorate many cardiac disorders, the hypothesis was that GJC modulation could have a similar outcome in bone tissue. Further, based on the complex signaling between bone cells, it was hypothesized that the presence of the osteoclastogenesis regulating MSCs could have an impact on the effects of GJC modulation. Concerning this, the aim of the study was also to compare the in vitro osteoclastogenesis of cultures containing the MSCs and in cultures lacking them.

The specific objectives of this study were to examine:

1. The importance of GJC in osteoclastogenesis.
2. The effects of stimulation of GJC in osteoclastogenesis.
3. The contribution of MSCs to the effects of GJC modulation.
4. The influence of the MSCs on osteoclastogenesis in vitro.
4 Materials and methods

4.1 Cell isolation and cultures

4.1.1 Cell lines

RAW 264.7 cells (I)

The murine monocyte-macrophage line RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in D-MEM (ATCC) containing 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4.5 g/l glucose and 1.5 g/l sodium bicarbonate (Sigma-Aldrich, St. Louis, MO). The cells were maintained in T25 or T75 cell culture flasks and passaged every 2-3 days. 6.3×10⁵ cells/cm² were cultured on sonicated bovine cortical bone slices (0.25-0.64 cm²; Lehenkari Consulting, Oulu, Finland) in 24 or 96-well plates (Cellstar; Greiner Bio-One, Monroe, NC). Osteoclastogenesis was induced with 50 ng/ml RANKL (PeproTech EC, London, UK). The cell culture medium was changed every 2-3 days and the cells were cultured at +37°C (5% CO₂, 95% air) for 8 days.

RAW 264.7 and MC3T3-E1 coculture (I)

In the coculture assay the RAW 264.7 cells were cultured with the murine preosteoblastic MC3T3-E1 cell line (LGC Standards, Teddington, UK). MC3T3-cells were maintained in α-MEM containing 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1% L-glutamine (Sigma-Aldrich) in T25 cell culture flasks and passaged every 2-3 days. In the coculture assay, MC3T3 cells (1×10⁴ cells/cm²) were first allowed to attach into 96-well plates (Cellstar; Greiner Bio-One) for 2-3 hours in α-MEM. After this, RAW 264.7 cells (2×10⁵ cells/cm²) in D-MEM (ATCC) were added to the wells. Osteoclastogenesis in RAW 264.7 cells was induced with 50 ng/ml RANKL (PeproTech EC). The cell culture medium was changed every 2-3 days and the cells were cultured at +37°C (5% CO₂, 95% air) for 8 days.
4.1.2 Primary cell cultures

Mouse bone marrow mononuclear cells (I)

8-12-week-old C57BL/6 mice were sacrificed with CO\textsubscript{2} inhalation and cervical dislocation. The study was approved by the Laboratory animal center at the University of Oulu, decision number 027/11. Femurs and tibias were dissected and rinsed in 70% ethanol and phosphate buffered saline (PBS). The bone ends were cut off and the bone marrow was rinsed out of the bones with α-MEM (GIBCO) containing 10% FBS, 2mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 20 mM Hepes buffer (Sigma-Aldrich) using a needle and a syringe. The cell suspension was centrifuged at 190×g for 10 minutes and suspended in α-MEM. The cells were plated on a petri dish and allowed to attach for 2 hours at +37 °C, after which the non-adherent cells were collected. Most of the MSCs in the bone marrow sample adhere to the plastic during the incubation, whereas the hematopoietic stem cells are non-adherent. However, some of the MSCs remain non-adherent, and therefore the bone marrow culture always contains some of these cells. The collected mononuclear cells were seeded on 96-well plates (3.1×10^6 cells/cm\textsuperscript{2}) on sonicated bovine cortical bone slices (0.28 cm\textsuperscript{2}; Lehenkari Consulting). The cells were cultured in α-MEM. Osteoclastogenesis was induced with 30 ng/ml RANKL (PeproTech EC) and 10 ng/ml M-CSF (R&D Systems, Minneapolis, MN). The cell culture medium was changed every 2-3 days, and the cells were cultured at +37°C (5% CO\textsubscript{2}, 95% air) for 8 days.

Human bone marrow mononuclear cells (II,III)

The bone marrow samples were obtained from patients undergoing hip replacement surgery in Oulu University Hospital. The patients were 79-85 (II) or 52-77 (III) – year-old men and women who gave a written informed consent for donation of the samples. The study was approved by the Ethical Committee of The Northern Ostrobothnia Hospital District, decision numbers 4/2000, 180/2001 and 29/2005, diary number 60/2011. The bone marrow sample was first cultured in α-MEM (Corning Life Sciences, Tewksbury, MA) containing 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin and 24 mM Hepes buffer (Sigma-Aldrich) at +37°C (5% CO\textsubscript{2}, 95% air) for 1-2 days. After the pre-culture, medium containing the non-adherent cells was collected and the isolation was continued with Ficoll-Paque Premium (GE Healthcare, Little Chalfont, UK). The cell suspension was
diluted 1:1 in PBS, layered over Ficoll-Paque Premium solution (1:1) and centrifuged at 400 x g for 35 minutes. The mononuclear cell layer was collected and washed twice by centrifugation at 190 x g for 10 minutes in PBS, and finally suspended in α-MEM (Sigma-Aldrich). 300 000 cells (9.4×10^5 cells/cm^2) were layered on sonicated human cortical bone slices (0.28 cm^2) in 96-well plates (Costar; Corning Life Sciences). The slices were cut from anonymous bone samples acquired from the clinical bone bank in Oulu University Hospital. The Special National Supervisory Authority for Welfare and Health (Valvira) granted permission for use of the cadaver specimens for research purposes, decision 8.5.2009, diary number 2240/05.01.00.06/2009. The cells were cultured in α-MEM containing 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich).

Osteoclastogenesis was induced with 20 ng/ml RANKL (PeproTech EC) and 10 ng/ml M-CSF (R&D Systems) (differentiation protocol referred to hereafter as RM) or with 50 ng/ml RANKL, 25 ng/ml M-CSF, 5 ng/ml TGF-β (R&D Systems) and 1 µM dexamethasone (Sigma-Aldrich) (referred to hereafter as RMTD). In study II, only the RMTD protocol was used for the bone marrow sample, but in study III both RM and RMTD were used. Half of the medium was refreshed every 3-4 days (100 µl). At the one week time point, all medium was refreshed (200 µl/well). Cells were cultured at +37°C (5% CO2, 95% air) for 14 days with RM and 12 days with RMTD.

Acidic culture conditions (II) were produced with a dropwise addition of 0.5 M HCl into the culture medium in order to lower the pH of the growth medium to 6.5 instead of physiological pH 7.4. The final concentration of HCl was between 4.5-10 mM. To maintain the pH, a final concentration of 20 mM Hepes (for pH 7.4) or 20 mM Pipes (for pH 6.5) buffer was used in the culture medium. Acidification was done in the beginning of the culture and after each medium change.

Human peripheral blood mononuclear cells (II, III)

Peripheral blood mononuclear cells were collected from whole blood samples from healthy donors or the hip replacement surgery patients shortly before the operation. The donors, 28-75 (II) or 52-77 (III) –year-old men and women, gave a written informed consent for donation of the samples. Mononuclear cell isolation for the diluted (1:1; PBS) blood sample was performed with Ficoll-Paque Premium gradient centrifugation as described earlier. Cell culture was done similarly to the bone marrow sample, and the same number of cells were cultured on the human
bone slices. Osteoclastogenesis and acidosis (II) were induced as described earlier. In study II, only the RM protocol was used for the peripheral blood sample, but in study III both RM and RMTD were used.

4.2 Modulation of GCJ (I, II)

Inhibition of GJC (I)

GJC was blocked with 1, 5 and 10 µM 18-α-glycyrrhetinic acid (18GA; Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and added to the cultures once daily. The final concentration of DMSO did not exceed 0.1% in any of the assays. The other GJC inhibitor, connexin mimetic peptide GAP27 (Zealand Pharma, Copenhagen, Denmark), was dissolved in water and added similarly to the cultures once daily to a final concentration of 5 or 500 µM.

Stimulation of GJC (I, II)

GJC was stimulated with four different gap junction modulating peptides (I): ZP250, ZP63, ZP178 and ZP1211 (Zealand Pharma; I). ZP63 is a pseudonym for AAP10 (H2N-Gly-Ala-Gly-4Hyp-Pro-Tyr-CONH2). Two scrambled (inactive) peptides, ZP1953 and ZP1954, were used as controls. The peptides were dissolved in water and added to the cultures once daily. The final concentrations of the peptides in the cultures were 0.1; 1; 10; 100 and 1000 nM. The active peptides were designed according to the pharmacophore model derived from the original structure-activity relationship data leading to the identification of rotigaptide. All peptides were synthesized according to the standard solid-phase procedure using the fluorenylmethyloxycarbonyl strategy.

In study II, only AAP10 was used for GJC stimulation and added to the cultures once daily to a final concentration of 100 µM.

Analysis of the effects of GJC modulation with fluorescence recovery after photobleaching (FRAP, II)

GJC was modulated with AAP10 in MC3T3-cells. The cells were maintained as described in chapter 4.1.1. For the FRAP experiment, 3.95x10^4 cells/cm^2 were cultured in 4 compartment glass bottom cell culture dishes (Cellview; Greiner Bio-
One, Kremsmünster, Austria) in 500 µl α-MEM per compartment. AAP10 was added to the cultures once daily at 100 µM concentration. The same concentration was added after staining with calcein red-orange. The cells were cultured for 3 days. The growth media were replaced with phenol red free D-MEM (Gibco/Thermo Fisher Scientific, Waltham, MA) containing 5 µM calcein red-orange, AM (Molecular Probes/Thermo Fisher Scientific). Staining was performed at +37°C for 30 minutes and the cells were washed 3 times with phenol red free D-MEM. FRAP experiments were performed with a Zeiss LSM 780 confocal microscope and Plan-Apochromat 20x/0.8 objective (Carl Zeiss Microscopy GmbH, Germany). The excitation wavelength was 561 nm and emission was collected between 566 nm - 670 nm. The pixel size was set to 0.69 µm x 0.69 µm. The time-lapse imaging interval was set to 5 seconds and recovery of the fluorescence was followed for 280 seconds. All measurements were performed at +37°C and 5% CO₂ maintained by an OkoLab bold line top stage incubator (OkoLab, Italy). Before analyzing the results, the fluorescence signals were corrected for acquisition bleaching and normalized by scaling the pre-bleach intensity values to 1, and the post-bleach values were scaled to 0.

4.3 Counting of multinuclear cells and the number of nuclei (I-III)

After culturing, the cells were fixed with 3% paraformaldehyde (PFA)/2% sucrose in PBS (I) or with 4% PFA in PBS (II-III) for 10 minutes at room temperature. The actin cytoskeleton of the cells was stained with fluorescein isothiocyanate (FITC)-conjugated phalloidin (0.1 mg/ml stock diluted 1:100-200 in PBS, Sigma-Aldrich; I) or with Alexa 488-conjugated phalloidin (200 U/ml stock diluted 1:100 in PBS; Invitrogen Europe, Paisley, UK; II-III) for 20 minutes at +37°C. The nuclei were stained with Hoechst 33258 (1 mg/ml stock diluted 1:800 in PBS; Sigma-Aldrich) for 10 minutes at room temperature. Staining of TRACP was performed with a commercial acid phosphatase leukocyte kit (Sigma-Aldrich) for 20 minutes at +37°C. The samples were mounted in 70% glycerol-PBS and viewed in a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan) and Plan 20 × (I) or 10 × (II-III) objective.

Multinuclear cells with three or more nuclei (I-III) and giant multinuclear cells with ten or more nuclei (I) were counted from each bone slice from five randomly chosen microscope fields, bone slice ≥3. In study I the number of nuclei were counted semiautomatically with a digital image analyzer (MCID Core 7.0 rev. 2.0, InterFocus Imaging, Linton, UK) coupled with a Retiga 4000R camera (QImaging,
Surrey, Canada) and Zeiss Axio Scope A1 microscope (Carl Zeiss, Oberkochen, Germany) with an Acroplan 20 × objective. The number of nuclei per osteoclast was evaluated by dividing the size of the group of nuclei by the average nuclear size. In study III the number of nuclei were counted from 4 multinuclear cells from 5 randomly chosen areas. Images were taken with a QImaging MicroPublisher 5.0 RTV camera and QCapture 2.90.1 software (QImaging). Confocal images (III) were taken with an LSM 510 META confocal microscope combined with an Axiovert 200 M inverted microscope (Carl Zeiss) with a 20 × Plan Neofluar objective (Carl Zeiss).

4.4 WGA-lectin staining and measurement of resorption pit areas (I)

The cells were detached from the bone slices by wiping the cellular surface of the slices with a soft brush. The resorption pits were stained with peroxidase-conjugated wheat germ agglutinin-lectin (WGA, 20 mg/ml; Sigma-Aldrich) for 20 minutes at room temperature. The samples were washed with PBS and incubated with diaminobenzidine (DAB) and hydrogen peroxide solution (DAB tetrahydrochloride substrate kit; Invitrogen/Life Technologies, Glasgow, UK) for 15 minutes at room temperature. The resorbed area was measured from 5 randomly chosen areas from each bone slice. Morphometric analysis of the resorption pits was performed with a Zeiss Axio Scope A1 microscope with an N-Acroplan 10 × objective and a digital image analyzer (MCID Core 7.0 rev. 2.0, InterFocus Imaging) coupled with a Retiga 4000R camera (QImaging).

4.5 Field emission scanning electron microscopy (FESEM; I-III) and measurement of resorption pit areas (II-III)

The samples were fixed with 2.5%glutaraldehyde in PBS for 3 hours (I) or with 4% PFA in PBS (II-III) for 10 minutes at room temperature.

For imaging with FESEM (I) the samples were dehydrated in an ascending ethanol series and dried in a Bal-Tec CPD 030 critical point drying unit (Bal-Tec Union, Balzers, Liechtenstein). Samples were coated with a 15 nm platinum layer in an Agar High Resolution Sputter Coater (Agar Scientific, Essex, UK). FESEM was performed in the secondary electron mode with a Zeiss Ultra Plus instrument using 5.0 kV voltage.
For resorption pit area measurements with FESEM (II-III) the cells were detached from the bone slices by brushing. The samples were dehydrated in an ascending ethanol series and dried with critical point drying equipment K850 (Quorum technologies, UK). Samples were coated with a 5 nm platinum layer by a Q150T ES sputter coater (Quorum Technologies) and viewed with a Sigma HD VP FESEM (Carl Zeiss). FESEM images were taken from three fields (voltage 5.0 kV, magnification 50x, area 0.035 cm$^2$) from each bone slice, n=3. The morphometric analysis of the pits was performed with ImageJ 1.49t software (NIH, USA) by superimposing a Merz grid with 80-88 points in semicircular lines over the image. Points in pits were counted and the proportion of resorption pits versus intact bone surface was counted (II-III). The proportion of resorbed area was normalized to cell number, and the average area resorbed by one cell was calculated (III).

4.6 Measurement of C-terminal telopeptide of type I collagen (ICTP; III)

The level of bone resorption can be quantitated by measuring the amount of ICTP fragments released into the culture medium. ICTP levels were measured with a UniQ ICTP radioimmunoassay kit (Orion Diagnostica, Espoo, Finland). The media were collected from human bone marrow and peripheral blood cultures on day 14 or 12 (depending on the culture protocol used; RM or RMTD). ICTP was measured according to the manufacturer’s instructions in duplicates from 3 cultures made from independent patient samples.

4.7 Transmission electron microscopy (TEM; I)

The samples were fixed with 2.5% glutaraldehyde in PBS for 3 hours at room temperature. Decalcification of the bone slices was performed with 5% EDTA (pH 7.4) for 7 days at +4 °C. The slices were further fixed with 2.5% glutaraldehyde in 0.1 M cacodyl buffer at pH 7.4, followed by contrast enhancement in 1% osmium tetroxide. The samples were embedded in EPON (TAAB Laboratories, Aldermaston, UK) and sectioned to ultrathin (50-100 nm) and semithin (0,4-1 µm) sections with an LKB ultramicrotome. The semithin sections were stained with toluidine blue. TEM photos were taken with a Philips Technai 10 electron microscope (Amsterdam, Netherlands).
4.8 Cx43 immunostaining (I)

For Cx43 immunostaining the cultured RAW 264.7 cells were fixed with 3% PFA/2% sucrose in PBS for 10 minutes at room temperature. The cells were permeabilized with 0.1% Triton X-100 for 10 minutes and blocked with 0.2% bovine serum albumin (BSA; Sigma-Aldrich) for 30 minutes at room temperature. Immunostaining was performed with mouse anti-Cx43 (0.5 mg/ml stock diluted 1:100 in PBS; Zymed, San Francisco, CA) for 2 hours at +37°C and Alexa Fluor 568 goat anti-mouse secondary antibody (2 mg/ml stock diluted 1:100 in PBS; Invitrogen/Life Technologies) for 30 minutes at +37°C. The actin cytoskeleton was stained with FITC-conjugated phalloidin (0.1 mg/ml stock diluted 1:100 in PBS; Sigma-Aldrich) for 20 minutes at +37°C. The samples were mounted in 70% glycerol-PBS and viewed in an LSM 510 confocal microscope (Carl Zeiss) combined with an Axiovert 100M inverted microscope with a 63 × objective (Carl Zeiss).

4.9 TRACP immunostaining (III)

For TRACP immunostaining mononuclear cells from BM and PB, samples were cultured on glass slides (0.20 cm²; 2.5x10⁶ cells/cm²) in 24-well plates (Costar; Corning Life Sciences). The cells were fixed with 4% PFA in PBS for 10 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes and blocked with 1% BSA (Sigma-Aldrich) for 30 minutes at room temperature. TRACP 5a was stained with 1:100 diluted rabbit serum anti-TRACP 5a for 1 hour at +37°C. TRACP 5a and 5b were stained with the same protocol with rabbit serum anti-TRACP, which binds to both 5a and 5b isoforms. TRACP antibodies were kindly donated by Göran Andersson (Karolinska Institutet, Stockholm, Sweden). The secondary antibody was goat anti-rabbit Alexa 488 (2 mg/ml stock diluted 1:100 in PBS; Invitrogen) incubated for 30 minutes at +37°C. The actin cytoskeleton was stained with tetramethylrhodamine-isothiocyanate (TRITC)-conjugated phalloidin (0.1 mg/ml stock diluted 1:100 in PBS; Sigma-Aldrich) for 20 minutes at +37°C. The samples were mounted in Immuno-Mount (Thermo Fisher Scientific, Waltham, MA) and viewed in an LSM 510 META confocal microscope combined with an Axiovert 200 M inverted microscope (Carl Zeiss) with a 10 × Plan Neofluar objective (Carl Zeiss).
4.10 Analysis of TRACP isoforms (III)

TRACP levels in culture media from human bone marrow and peripheral blood samples were measured with a sandwich ELISA for TRACP 5a and TRACP 5b separately. For capturing of TRACP 5a, media and recombinant TRACP 5a standards were diluted 1:20 in 0.1% BSA (Sigma-Aldrich) in TBST (25mM Tris pH 7.4, 150mM NaCl, 0.1% Tween-20) and added to 96-well half-volume ELISA plates (Costar, Chicago, IL) coated with 5 μg/ml mouse anti-TRACP 5a monoclonal antibody (mAb) 46 (Mabtech, Nacka, Sweden) at +4 °C overnight. The samples were incubated at +4 °C overnight. mAb 46 is developed to recognize only TRACP 5a. The supernatant was diluted 1:100 and transferred to 96-well half-volume ELISA plates (Costar) coated with 5 μg/ml mouse anti-TRACP (5a and 5b) mAb 25.44 (Mabtech) at 4 °C overnight for capturing TRACP 5b. All samples were incubated for 2h at room temperature, after which both plates (mAb 46 coated plate with captured TRACP 5a and the mAb 25.44 plate with the captured TRACP 5b) were washed 3 times in TBST. The plates were incubated with biotinylated mouse anti-TRACP (5a and 5b) mAb 12.56 (Mabtech) 0.25 μg/ml for 1h at room temperature, after which the plates were washed 3 times in TBST followed by incubation in Streptavidin HRP (Mabtech) diluted 1:1000 in 0.1% BSA in TBST. After incubation the plates were washed 3 times in TBST. Finally, the plates were washed and developed with K-Blue Substrate (tetramethylbenzidine; Neogen, Lansing, MI) for 20 minutes and absorbance was measured at 450nm in BioTek’s PowerWave HT microplate spectrophotometer (BioTek, Winooski, VT). Quantification of TRACP 5a was performed using the TRACP 5a standard curve on the mAb 46 coated plate. Quantification of TRACP 5b was performed using the newly added TRACP 5a standards and the absorbance of the supernatant on the plate coated with mAb 25.44. The transferred standards of TRACP 5a from mAb 46 to mAb 25.44 plate were used to correct for any carry-over of TRACP 5a from plate mAb 46 to plate mAb 25.44.

4.11 Quantitative polymerase chain reaction (qPCR; III)

After the cell culture, total RNA was isolated from the bone slices with a commercial RNA isolation kit (Macherey-Nagel, Düren, Germany). After media removal from the wells, lysis buffer with 20 mM dithioerythritol was pipetted back and forth in the wells and isolation was continued with the NucleoSpin RNA column purification. Lysis buffer from 3 parallel wells were combined in one
column. RNA concentration was measured with a Nano-Drop ND 1000 spectrophotometer. A Thermo Scientific First Strand cDNA Synthesis kit with both Oligo (dT)18 and D(N)6 primers was used for the reverse transcription of the RNA (Thermo Scientific). The PCR reactions were performed with 2 µl cDNA in a total volume of 20 µl of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) containing SYBR Green I dye, hot-start iTaq DNA polymerase, dNTPs and MgCl₂. The QPCR reaction was performed on a Bio-Rad CFX Connect device with the following program: activation step (10 min, 94°C) and 40 cycles of two-step PCR (95°C for 15 seconds, 60°C for 1 minute). For the TATA-box binding protein (TBP) housekeeping gene a three-step protocol was performed (95°C for 10 seconds, 55°C for 30 seconds, 72°C for 30 seconds). Two endogenous controls, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TBP housekeeping genes were used for normalization of the samples. The housekeeping genes were shown to be stable using Bio-Rad CFX Manager Gene Study, version 3.1. The primers for the housekeeping genes and targets (calcitonin receptor; CR, and nuclear factor of activated T-cells; NFATc1) synthesized by Metabion International (Planegg, Germany) are listed in table 1. Unspecific PCR products were tested by subjecting all genes to melt curve analysis. QPCR analysis was performed for cultures obtained from 3 independent donors.

Table 1. qPCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: ATGGGGAAGGTGAAGGTCG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TAAAAACAGCCCTGGTGACC</td>
</tr>
<tr>
<td>TBP</td>
<td>Forward: GGTCCTGGGAAAATGGTGTGC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTGAAAAACCCAACTCTG</td>
</tr>
<tr>
<td>CR</td>
<td>Forward: GCATACCAAGGAGAAGGTCCATAT</td>
</tr>
<tr>
<td></td>
<td>Reverse: ATACTCCAGCAGGGTGCTCAT</td>
</tr>
<tr>
<td>NFATc1</td>
<td>Forward: AGCAGAGCGCCAGAGGAGTCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGTCAGTTCTCGCTTCCATCT</td>
</tr>
</tbody>
</table>

4.12 Statistical analysis (I-III)

All experiments were done with groups of n≥3 and repeated with at least 3 independent patient samples (II,III) or at least 2 cell culture experiments with the cell lineages (I). Statistical analyses were performed using an SPSS statistics program version 20 (I) or 22 (II,III; SPSS Inc., Chicago, IL). The normality of the response variables was tested with Kolmogorov-Smirnov or Shapiro-Wilk test (for
experiments with n<50) and histogram visualization. If the response variables were
normally distributed, statistical differences between the test groups were evaluated
using one-way ANOVA, and comparison between groups was done with a two-
sample t-test (II,III). In study I Dunnett’s post hoc test was used for comparison to
control cultures, and multiple comparisons between the other samples were done
with Sheffe’s post hoc test. If the response variables were not normally distributed,
statistical differences between the test groups were evaluated using the Kruskal-
Wallis test, and comparison between groups was done with the Mann-Whitney U-
test. The graphical presentation of the results was created with OriginPro 8.6 (I) or
9.1 (II, III) software (OriginLab, Northampton, MA). p<0.05 was considered
significant. Data are shown as means ± SEM.
5 Results

5.1 Blocking of GJC reduces osteoclastogenesis (I)

The effect of GJC blocking on osteoclastogenesis was investigated in study I. Osteoclastogenesis in murine monocyte-macrophage line RAW 264.7 cells was induced with RANKL and the cells were treated with 1, 5 and 10 µM concentrations of the gap junction blocker 18GA. As a result, the number of multinuclear cells decreased significantly at the 10 µM concentration whereas the proportion of multinuclear giant cells with 10 or more nuclei decreased already at the 5 µM concentration. In hematopoietic cells isolated from mouse bone marrow and differentiated into osteoclasts with RANKL and M-CSF, 18GA reduced the number of multinuclear cells at all concentrations used (1, 5 and 10 µM) and also decreased the resorption pit area at 1 and 10 µM concentrations.

The specific GJC blocker GAP27 decreased the number of multinuclear RAW 264.7 cells at 5 and 500 µM concentrations, but did not have an effect on the number of nuclei per cell.

The results from GJC blocking indicate that gap junctions are important mediators in the fusion of the cells, but since the fusion was not totally blocked, there are also other fusion mechanisms to compensate for the non-functional GJC.

5.2 Stimulation of GJC increases osteoclastogenesis (I, II)

The stimulation of GJC during osteoclastogenesis was investigated in studies I and II. In study I, four novel AAPs were used during differentiation of RAW 264.7 cells into osteoclasts with RANKL. All AAPs increased the number of multinuclear cells at 0.1-1000 nM concentrations compared to the control group. The inactive peptides used as controls did not have a significant effect on the multinuclear cell number, albeit the cell number in the treated groups fluctuated slightly above and below the control values.

In study II the stimulation of GJC was investigated with the most potent AAP used in study I, AAP10, in osteoclastogenesis of monocytes isolated from human bone marrow and peripheral blood. At pH 7.4 AAP10 increased the number of multinuclear cells as well as bone resorption area significantly in both bone marrow and peripheral blood cultures.
The results show that osteoclastogenesis and bone resorption can be enhanced by stimulation of GJC at physiological pH.

5.3 The effects of AAPs on bone cells are mediated through gap junctions

5.3.1 Gap junction-like connections between multinuclear cells (I)

In study I, TEM analysis of mouse bone marrow cultures and RAW 264.7 cultures revealed gap junction-like connections between the cells. First, in RAW 264.7 cultures some mononuclear cells seemed to be in contact with a multinuclear cell with a gap junction-like structure. Second, in mouse bone marrow cultures, some multinuclear cells seemed to be similarly connected to each other. This indicates that both the fusing mononuclear cells as well as the already fused multinuclear cells might communicate via gap junctions.

5.3.2 AAPs induce morphological changes in Cx43 localization (I)

Cx43 was immunostained in RAW 264.7 cells in study I. Although not unquestionably proven, there was a tendency towards increased accumulation of Cx43 in the cell contacts between the mononuclear cells after treatment with the AAPs. The staining was faint upon addition of the GJC blocker 18GA. The results support the idea that the AAPs might increase Cx43 expression in mononuclear RAW 264.7 cells.

5.3.3 AAP10 improves fluorescence recovery after FRAP (II)

The effects of AAP10 on GJC between osteoblast lineage MC3T3 cells were investigated in study II. FRAP analysis showed that AAP10 increased the recovery of fluorescence after photobleaching indicating that AAP10 affects GJC in these cells. FRAP analysis is based on the diffusion of molecules through gap junctions, therefore, the increased recovery with AAP10 is evidence of increased GJC.
5.4 MSCs in the osteoclast culture contribute to osteoclastogenesis

5.4.1 The presence of MSCs varies between different monocyte source tissues (I-III)

The several monocyte sources in the experiments of this study differed in the presence of MSCs. The RAW 264.7 cell line is a pure cell line in a sense that it contains solely the monocyte-macrophage lineage cells. The peripheral blood derived monocyte cultures resemble the RAW 264.7 cultures as blood contains only few MSCs. In contrast to these cultures, both mouse and human bone marrow monocyte cultures contain always an abundance of MSCs as well as other mononuclear cells from the bone marrow. The thin and elongated fibroblast-like cells of the bone marrow are hereafter referred to as MSCs based on their differing morphology compared to the spherical monocytes. In addition, while the mononuclear monocytes often stain TRACP-positively even before the cell fusion into multinuclear osteoclasts, the MSCs are always TRACP-negative, supporting the idea that these cells have a mesenchymal origin. The isolation of the hematopoietic cells from the bone marrow sample is based on the adherence of the MSCs on the plastic surface, but since some of these cells remain non-adherent, their presence in the cultures cannot be avoided completely. The presence of the MSCs in the cell cultures of this study is shown in fig 7.
5.4.2 The effects of AAPs are influenced by the MSCs in mouse bone marrow culture (I)

In study I the AAPs were shown to increase the number of multinuclear RAW 264.7 cells. However, in mouse hematopoietic stem cells the effect was opposite, as the AAPs decreased the number of multinuclear cells at all concentrations from 0.1 to 1000 nM compared to the control group. Similar to RAW 264.7 cell cultures, the inactive peptides did not have an effect on the multinuclear cell numbers.
The results show that the effect of the stimulation of GJC depends on the type of cell culture investigated, and the probable players causing the different effects could be the MSCs present in the bone marrow cultures. This hypothesis is supported by the fact that the effects of the AAPs were different in the RAW 264.7 cell culture containing only the monocyte-macrophage lineage cells compared to the heterogeneous bone marrow cultures containing the MSCs and the other mononuclear cells from the bone marrow.

5.4.3 The presence of osteoblast-lineage MC3T3 cells dispels the osteoclastogenesis promoting effect of AAP10 in RAW 264.7 cells (I)

To further investigate the possible contribution of the MSCs in the effects of GJC modulation in osteoclastogenesis, a coculture of RAW 264.7 cells and osteoblast lineage MC3T3 cells was established in study I. In the coculture, MC3T3 cells seemed to act like the MSCs in the bone marrow cultures, since the osteoclastogenesis increasing effect of AAP10 was abolished in the coculture. This indicates that the MSCs contribute to the net effect of GJC modulation during osteoclastogenesis.

5.4.4 The effects of AAPs in acidosis are different in cultures containing and lacking MSCs (II)

The effects of the MSCs in osteoclastogenesis were also investigated in study II, where GJC was modulated with AAP10 in acidosis. Acidosis was studied based on the fact that AAPs have been reported to be more efficient under environmental stress such as acidosis, and, in addition, rotigaptide has been shown to impair the acidosis induced gap junction closure. Further, osteoclastogenesis and bone resorption are enhanced in acidosis, which makes the condition suitable for osteoclastogenesis study.

Acidosis significantly increased the number of multinuclear cells as well as bone resorption in human bone marrow monocyte cultures. In peripheral blood monocyte cultures only bone resorption was increased in acidosis.

When AAP10 was added to the cultures in acidosis, it had no effect on the multinuclear cell number or bone resorption in the bone marrow cultures. In peripheral blood cultures the addition of AAP10 in acidosis decreased both multinuclear cell number and bone resorption. Since the two cultures differ in the
presence of the MSCs, the explanation could be that GJC modulation also affects the gap junctions in the MSCs. Therefore, the net effect on osteoclastogenesis is dependent also on the secretion of osteoclastogenesis regulating factors from the MSCs, which are activated by GJC stimulation.

5.5 The monocytes in bone marrow and peripheral blood have a differing capacity for osteoclastogenesis

After the observation that the MSCs had an influence on the effect of GJC modulation, we further analyzed the effect of the MSCs in osteoclastogenesis. We compared osteoclastogenesis from human peripheral blood and bone marrow monocytes, since these cultures clearly differ in the presence of MSCs. As described earlier, peripheral blood contains only a few MSCs, whereas a higher number are present in the bone marrow monocyte cultures.

5.5.1 Osteoclastogenesis induced with RANKL and M-CSF (III)

When osteoclastogenesis in bone marrow and peripheral blood derived monocytes was induced with RANKL and M-CSF, both cell types differentiated into multinuclear TRACP-positive cells. The number of multinuclear cells was higher in the bone marrow sample. However, there were no significant differences between the two monocyte sources in the number of nuclei per osteoclast, bone resorption activity, secretion of TRACP isoforms 5a and 5b or the expression of osteoclast specific genes CR and NFATc1. The results show that the greater population of MSCs present in the bone marrow samples did not have major effects on osteoclastogenesis when it was induced with the two essential growth factors, RANKL and M-CSF.

5.5.2 Osteoclastogenesis induced with RANKL, M-CSF, TGF-β and dexamethasone (III)

Peripheral blood derived osteoclasts contained more nuclei

When TGF-β and dexamethasone were used in addition to RANKL and M-CSF for induction of osteoclastogenesis, we observed differences between bone marrow and peripheral blood monocytes. The number of multinuclear cells in the peripheral
blood cultures was slightly higher compared to bone marrow cultures, but the effect was not statistically significant. However, the number of nuclei in the multinuclear cells was significantly higher in peripheral blood derived osteoclasts. The difference in the number of nuclei did not lead to differences on the bone resorption activity of the osteoclasts.

*Peripheral blood derived osteoclasts expressed higher levels of TRACP 5a and 5b*

The expression of TRACP isoforms was analyzed by immunofluorescence and ELISA methods. Immunofluorescence showed that the intracellular TRACP 5a expression did not differ between the osteoclasts derived from bone marrow and peripheral blood. However, ELISA revealed that significantly more TRACP 5a was secreted into the cell culture medium from peripheral blood derived osteoclasts. Similarly, TRACP 5b was secreted in higher levels from peripheral blood derived osteoclasts, and immunostaining with an antibody binding to both TRACP 5a and 5b was more intense in peripheral blood derived osteoclasts. The higher level of TRACP 5b expression and secretion from peripheral blood cultures probably correspond to the higher number of multinuclear cells in these cultures. Concerning the higher secretion levels of TRACP 5a from the peripheral blood cultures, it was noticed that these cultures contained more mononuclear cells corresponding to the secretion of the macrophage type TRACP 5a.

*The expression of osteoclast-specific genes was higher in peripheral blood derived osteoclasts*

Although the results were not statistically significant, we observed a tendency towards higher expression of osteoclast specific genes *CR* and *NFATc1* in the osteoclasts derived from peripheral blood. The expression of these genes in both bone marrow and peripheral blood derived osteoclast cultures indicates that monocytes from both cell sources had differentiated into osteoclasts. It is possible that the peripheral blood derived cells presented a more complete osteoclast differentiation profile indicating their increased response into TGF-β and dexamethasone and a higher osteoclastogenic capacity.
6 Discussion

Although gap junctions can be found in cells throughout the body and are shown to have various roles during development, differentiation and cell growth, their role in the sequential cell fusions during osteoclastogenesis is less known. Gap junctions have been shown to be expressed in osteoclasts (Ilvesaro et al., 2000; Schilling et al., 2008), and GJC inhibition has been shown to decrease osteoclastogenesis and bone resorption (Ilvesaro et al., 2000; Ilvesaro et al., 2001; Matemba et al., 2006; Ransjö et al., 2003; Schilling et al., 2008). In addition to the direct effects of GJC inhibitors on the osteoclast precursor cells, GJC modulation in the osteoblast lineage cells can indirectly regulate osteoclastogenesis (Watkins et al., 2011; Y. Zhang et al., 2011). This is an interesting finding since the osteoblast lineage/MSCs are important regulators of osteoclastogenesis and osteoclast functions. Therefore, we have studied the role of GJC in osteoclastogenesis from mouse and human mononuclear cells of bone marrow and peripheral blood origin. Since these sources differ in the presence of MSCs, we hypothesized that these cells could be the mediators behind the effects of GJC modulation in osteoclast precursors. Secondly, we wanted to study the contribution of the MSCs to osteoclastogenesis from these different monocyte sources.

6.1 GJC is involved in osteoclastogenesis

In this study, blocking of GJC with 18GA and GAP27 in murine monocytemacrophage RAW 264.7 cells and in mouse hematopoietic cells decreased the number of multinuclear cells, which indicates that GJC is utilized during cell fusion in osteoclastogenesis. The observation that 18GA decreased the number of giant multinuclear RAW 264.7 cells with more than 10 nuclei already at a lower concentration suggests that GJC is probably involved in the later stages of the fusion process. The results are in line with studies showing that osteoclasts in mice with a Cx37 deletion are smaller and have fewer nuclei (Pacheco-Costa et al., 2014) and that blocking of GJC with heptanol leads to a lower number of nuclei in osteoclasts (Schilling et al., 2008). On the contrary, Schilling et al. showed that Cx43 mRNA expression decreased time-dependently during the course of differentiation indicating that GJC is involved in the early fusion of the mononuclear precursors into osteoclasts. However, in their study, Cx43 mRNA levels were measured until differentiation day 28, and the expression seemed to be quite stable until day 14. In addition, they found that Cx43 immunostaining was
stronger in osteoclasts with fewer than three nuclei compared to the large osteoclasts cultured for 28 days. Together these data suggest that GJC is involved in osteoclastogenesis probably at stages when some small multinuclear cells already exist, and the surrounding mononuclear cells are fusing into these. Additionally, since some small multinuclear cells always exist in the cultures with the GJC inhibitors, GJC is neither the first nor an indispensable step in cell fusion and the beginning of the fusion is probably mediated by the other fusion proteins. For instance, CD47 and syncytin have been shown to regulate the fusion of two mononuclear precursors and the fusion of two multinuclear osteoclasts, respectively (Møller et al., 2017). Further evidence supporting the idea that GJC is involved in the fusion of mononuclear cells into small multinuclear cells, Schilling et al. found that without RANKL in the cell culture, Cx43 expression started to decrease already at day 3. This suggests that GJC occurs downstream of RANKL and that RANKL might enable the long-lasting expression of Cx43 allowing GJC during osteoclastogenesis.

In our study, blocking of GJC with 18GA in mouse hematopoietic cells affected also bone resorption by decreasing the resorbed area. This effect is also noted in the other studies with GJC inhibitors (Ilvesaro et al., 2000; Ilvesaro et al., 2001; Ransjö et al., 2003), but none of these studies have examined whether the effect is due to the decreased osteoclastogenesis and therefore fewer osteoclasts to resorb the bone, or a direct regulation of the osteoclasts’ resorptive activity. Both options are possible, since osteoclastogenesis and bone resorption can be differentially regulated (Fuller, Kirstein, & Chambers, 2006). In their study, Fuller et al. demonstrated that RANKL, the crucial osteoclastogenesis-inducing molecule, can also activate the mature osteoclasts for bone resorption. Similarly, it is possible that GJC occurs during osteoclastogenesis as well as between the mature osteoclasts undergoing bone resorption.

Furthermore, neither the earlier studies nor ours can conclude whether the inhibiting agents affect the gap junctions between the fusing osteoclast precursors or the hemichannels between these cells and the extracellular matrix, where the substances secreted by MSCs and other cells enter the scene. It is also possible that the osteoclast precursors and MSCs are in direct contact by gap junctions allowing the rapid exchange of the regulatory molecules. Watkins et al. (Watkins et al., 2011) and Zhang et al. (Y. Zhang et al., 2011) have provided evidence of the contribution of MSCs in the modulation of GJC in bone tissue showing that Cx43 deletion in the osteoblast lineage cells leads to lower levels of OPG production, thereby indirectly promoting osteoclastogenesis. In addition, Furuya et al. (Furuya et al.,
2018) recently demonstrated that mature osteoblasts can inhibit bone resorption through a direct contact to osteoclasts, although the structures mediating the signaling were not characterized in this study. It is possible that GJC also participates in this type of signaling. Another study by Li et al. (Z. Li et al., 2018) demonstrated that glycyrrhizin, a glycoside isolated from licorice root and closely resembling the glycyrhetinic acid (18GA) used in our study, inhibited osteoclastogenesis in mouse bone marrow-derived monocytes by downregulating NFATc1, c-fos, TRACP, cathepsin K, DC-STAMP and OSCAR. Although the authors do not discuss the role of GJC or the bone marrow-derived MSCs in the effects, it is possible that the effects are mediated by GJC either as a direct action on the monocytes or indirectly through the MSCs as described above.

In conclusion, our results favor the idea of the direct effects of GJC inhibiting agents on osteoclast precursors, since similar results of decreased osteoclastogenesis were observed in the pure monocytic RAW 264.7 cell cultures as well as in the mouse bone marrow cultures also containing MSCs.

6.2 Osteoclastogenesis can be stimulated with AAPs

Although several GJC inhibitory agents are available, AAPs are currently the only specific stimulators of GJC. Their effects are extensively studied in heart tissue, since according to their nomenclature, they have promising clinical value in the treatment of various heart dysfunctions. Since the development of the AAPs, several clinical trials have been performed in order to develop new drugs for cardiac diseases. Regarding orally administered drugs, side effects in the other tissues of the body must be taken into account. The functions of the AAPs in bone tissue have remained unknown, therefore, we have studied their role in osteoclastogenesis and bone resorption.

We observed that stimulation of RAW 264.7 cells with four novel AAPs increased the number of multinuclear cells. The stimulatory effect was confirmed by control experiments with inactivated AAPs, which did not have an effect on cell fusion. The minor fluctuations in the multinuclear cell number seen with the inactive AAPs likely represent the biological variation in the osteoclastogenic capacity between the individual animal samples used for the study.

For the subsequent studies, the most potent peptide, AAP10, was chosen. AAP10 stimulated osteoclastogenesis and bone resorption in monocytes derived from human bone marrow and peripheral blood at physiological pH. The results show that AAPs can be used for stimulation of osteoclastogenesis from monocytic
precursors of both mouse and human origin. However, the detailed mechanisms behind the increased osteoclastogenesis upon the addition of AAPs remain to be discovered. AAPs have been reported to exert their effects either by increasing Cx43 expression (Stahlhut et al., 2006) or by altering the PKC-mediated phosphorylation of Cx43 (Axelsen et al., 2006; Weng et al., 2002). We demonstrated gap junction-like connections between mononuclear RAW 264.7 cells and between multinuclear cells in the mouse bone marrow cultures, as well as the possible increased accumulation of Cx43 in RAW 264.7 cells upon treatment with the AAPs. These results support the idea of increased Cx43 expression behind the effects of AAPs. Further, we demonstrated that AAP10 improved the recovery of fluorescence in the osteoblast lineage MC3T3 cells in the FRAP experiment, indicating that AAP10 exerts its actions via the gap junctions. Based on these results, it is possible that AAP10 acts by both mechanisms: by increasing the total Cx43 expression leading to higher numbers of gap junctions present on the cell surface, as well as by improving the probability that the gap junctions are open.

As mentioned in the previous chapter concerning the mechanisms behind GJC inhibition, we cannot draw conclusions on whether AAPs increase the signaling between the fusing mononuclear cells or the signaling between the cells and the extracellular milieu via the hemichannels. The results from the pure RAW 264.7 monocyte cultures support the direct actions of the AAPs on the cells, but the results described in the following chapter concerning the different effects obtained with the mouse bone marrow cultures and the acidosis model with human monocytes suggest the possibility of indirect actions via the MSCs.

Of note in interpreting the results from the RAW 264.7 cell line cultures is the fact that they represent an immortalized and transformed cell line with characteristics that are not fully known. Although multinuclear cells with actin rings were formed from the mononuclear RAW 264.7 cells, their resorptive activity was relatively poor. RAW 264.7 cells have been used in many osteoclast studies and shown to express several osteoclast markers (Cuetara, Crotti, O'Donoghue, & McHugh, 2006; Gu et al., 2015), but their resorptive activity is mainly analyzed on dentine or calcium phosphate disks rather than on genuine bone surfaces (Cuetara et al., 2006; Ren et al., 2017; Vincent, Kogawa, Findlay, & Atkins, 2009; Q. Xiong et al., 2015). These studies, as well as our observation of the poor resorptive activity of RAW 264.7 cells, raise the question of whether they can be considered as “real” osteoclasts. Even though their resorptive activities would be shown unquestionably, they are still derived from a cell line and possibly altered during the culture passages and maintenance on plastic culture surfaces. Thus, they are suitable for
the preliminary screening of substances affecting osteoclastogenesis, but clearly do not represent the cells present in tissues in vivo.

Another issue to be considered in developing the GJC affecting drugs is the fact that relatively high concentrations of both GJC blockers and stimulators are needed to achieve their required effects. In our study the inhibitory effects of 18GA and GAP27 were observed at concentrations between 1-10 µM and 5-500 µM, respectively. Although the concentrations of 18GA were quite low, there was no linearity in the effects in the mouse hematopoietic cell cultures with an increasing concentration. Similar results without the linearity were observed with the AAPs in both RAW 264.7 cell and mouse hematopoietic cell cultures. In human monocyte cultures, AAP10 was used at a 100 µM concentration since no effects were seen with lower concentrations. The need for the high concentration has been observed with both the GJC blockers and stimulators (Ilvesaro et al., 2000; Ilvesaro et al., 2001; Ponsaerts et al., 2010; Schilling et al., 2008). It has been suggested that the high concentrations are required for the transfer of the molecules into cells and for their intracellular distribution to reach the reactive sites with gap junctions (Iyyathurai et al., 2013). Moreover, the GJC modulators might not bind to their targets with a high affinity, and secondly, their half-life might be very low. For instance, AAP10 was shown to have a half-life of about 4 and 12 minutes in rat and human plasma, respectively (Kjølbye et al., 2003). Since the half-life of AAP10 in cell culture is not known, we chose to add it daily to the cultures, and it is possible that the high concentrations needed to achieve the effects are due to the low half-life of the peptide. Considering the low half-life and the high concentrations of AAPs needed in in vitro studies, problems might arise in designing the therapeutic doses of these compounds. The therapeutic dose of a drug is dependent on various factors and complex events occurring inside the body ranging from administration to distribution of the drug to the target tissue and the final metabolism at the preferred site. In the case of peptide drugs, one of the biggest challenges in the development is their degradation in the gastrointestinal tract, which usually leads to the requirement for improvement in peptide stability (Fosgerau & Hoffmann, 2015).
6.3 MSCs contribute to GJC in osteoclastogenesis

6.3.1 GJC stimulation in MSC-containing cultures can lead to either inhibition or stimulation of osteoclastogenesis

Although we showed that AAPs can be used for stimulation of GJC in osteoclast precursors leading to increased osteoclastogenesis, differences occurred in certain cultures containing also MSCs. First, when mouse hematopoietic cells were treated with the four different AAPs during osteoclastogenesis, all AAPs decreased the number of multinuclear cells. The results were confirmed with the inactive peptides, which did not have an effect on cell fusion. We hypothesized that the decreased osteoclastogenesis observed in these cultures could be mediated by the stromal/osteoblast lineage cells. To study this, we created a coculture with the mouse monocyte/macrophage lineage RAW 264.7 cells and osteoblast lineage MC3T3 cells, which are osteogenic cells capable of differentiation into mineralizing osteoblasts (Sudo, Kodama, Amagai, Yamamoto, & Kasai, 1983).

In the coculture the AAPs lost their osteoclastogenesis-promoting effects which were observed when only RAW 264.7 cells were studied, suggesting that the stimulation of GJC in the osteoblast lineage cells with AAPs induces them to produce osteoclastogenesis-inhibiting substances. As discussed above, this indirect regulation of osteoclastogenesis is indeed the possible mechanism of action of the AAPs. However, as discussed regarding the cell line cultures with RAW 264.7 cells, these cocultures created from two cell lineages are far from the situation in vivo, where dozens of other cell types participate in the signaling through several complex pathways. Therefore, in the subsequent studies we examined the effects of AAPs in primary human cells, since the primary cultures better represent the in vivo situation and because clear conclusions concerning human tissues cannot be made from mouse cell cultures.

As discussed above, at physiological pH we did not observe differences in the GJC stimulatory effects of AAP10 in bone marrow and peripheral blood derived...
monocyte cultures, as AAP10 increased multinuclear cell number and bone resorption in both cell types. As shown in the results section, the presence of MSCs differed greatly between these sources. MSCs were hardly seen in peripheral blood cultures, whereas in bone marrow cultures they existed in large quantities creating thin patches over the bone slice. Moreover, the MSCs were sometimes seen covering the resorption pits similar to the canopy cells covering the BRC (Hauge et al., 2001). We characterized these cells only by TRACP staining, which revealed that these spindle-like cells were always TRACP-negative indicating their mesenchymal origin. It is probable that these cells represent the many types of stromal cells in bone marrow (Klimczak & Kozlowska, 2016).

6.3.2 MSCs contribute to the effects of GJC modulation during osteoclastogenesis in acidosis

Since it has been reported that the AAPs could be more active in acidosis (Eloff et al., 2003), our hypothesis was that acidosis could enhance the osteoclastogenesis-stimulatory effects of AAPs. The acidosis model is interesting in regard to osteoclast studies, since osteoclastogenesis and bone resorption are activated in acidosis (Ahn, Kim, Lee, Kim, & Jeong, 2012; Arnett, 2008; Kato & Morita, 2011; Meghji, Morrison, Henderson, & Arnett, 2001). Secondly, acidosis can lead to closure of the gap junctions (Spray, Harris, & Bennett, 1981), which rotigaptide has been reported to dissipate (Eloff et al., 2003).

In our study, acidosis increased osteoclastogenesis and bone resorption in human bone marrow derived monocyte cultures. In peripheral blood derived cultures only resorption was enhanced indicating that a distinct mechanism is probably behind the effects of acidosis in these cultures. Acidosis has been reported to directly enhance osteoclast survival, adhesion and migration (Ahn et al., 2012) and to increase the expression of genes required for bone resorption (Arnett, 2008). However, also osteoblasts contain proton receptors (Ludwig et al., 2003), therefore, acidosis affects also osteoblasts. For instance, acidosis was reported to inhibit matrix mineralization by osteoblasts (Brandao-Burch, Utting, Orriss, & Arnett, 2005). Therefore, while Fuller et al. described that osteoclastogenesis and bone resorption can be differentially regulated, they also remarked that the contribution of osteoblast lineage cells in the effects of acidosis have not been taken into account (Fuller et al., 2006). Many studies of acidosis and osteoclastogenesis were made with bone marrow cell cultures, which likely contain also the osteoblast lineage cells to some degree. Thus, it is possible that the indirect regulation of
osteoclastogenesis by the osteoblast lineage cells causes some of the effects of acidosis. A few studies have provided evidence for this as osteoblasts were found to secrete osteoclast-activating substances such as prostaglandins and RANKL in acidosis (Frick & Bushinsky, 2003; Krieger, Parker, Alexander, & Bushinsky, 2000). In conclusion, the presence of the MSCs in the bone marrow sample is likely to be one explanation for the increased osteoclastogenesis during acidosis, which was not observed in the peripheral blood cultures.

Another explanation for the differences in osteoclastogenesis and bone resorption observed between the bone marrow and peripheral blood cultures could be the differing monocyte pools in these sources. Sprangers et al. suggested that the classical monocytes could be the osteoclast precursors in physiological conditions, whereas the intermediate monocytes would be recruited for osteoclastogenesis during inflammation (Sprangers et al., 2016) along with a higher bone resorption activity. This, and the fact that the main monocyte type in blood is the classical monocyte while bone marrow contains mostly intermediate monocytes support our results, as the bone marrow derived osteoclasts were more active in bone resorption. Acidosis could well be the stressor causing the differentiation of the intermediate monocytes in the bone marrow cultures leading to the high resorption activity of the generated osteoclasts.

When acidosis and treatment with AAP10 were combined during osteoclastogenesis, differences were observed between cultures from human bone marrow and peripheral blood. Although acidosis increased osteoclastogenesis and bone resorption in bone marrow culture, the addition of AAP10 had no effect on either process. In peripheral blood cultures AAP10 decreased both the number of multinuclear cells and bone resorption in acidosis. We believe that the explanation for these results lies in the prominent MSC population in our bone marrow preparation. First, it is possible that acidosis induces the closure of gap junctions. AAP10 does not seem to be able to maintain the open configuration, and therefore is not functioning in a similar way to rotigaptide in acidosis (Eloff et al., 2003). The increased osteoclastogenesis observed in bone marrow cultures proceeds possibly through other compensating mechanisms without the need for GJC. Moreover, the MSCs in the bone marrow cultures can produce various osteoclastogenesis-promoting molecules. Secondly, it is possible that AAP10 affects GJC in the MSCs further increasing the production of the molecules regulating osteoclastogenesis. Jørgensen et al. have shown evidence that this might be the mechanism of how AAPs act in bone tissue, since they demonstrated that rotigaptide increased GJC in human osteoblasts both during normal conditions as
well as during metabolic stress (Jørgensen et al., 2005). Since the peripheral blood cultures contain very few MSCs, their supportive effect on osteoclastogenesis during acidosis is missing, leading to decreased numbers of osteoclasts.

Overall, the results from culture under normal and acidic pH conditions reveal differences in the effects of AAPs on bone cells depending on the homeostatic state of the tissue. The complex signaling pathways in the bone tissue and the fact that GJC and inflammation are linked to each other create challenges in developing GJC-regulatory drugs either for the cardiac symptoms or the bone diseases. Inflammation can lead to increased or decreased GJC (Willebrords et al., 2016), while inflammation and bone cell functions are associated in the various osteoimmunological processes (Zupan et al., 2013). Acidosis further complicates the issue, as bone resorption creates an acidic environment under the osteoclasts while also inflammation can cause acidosis (Arnett, 2010). Secondly, acidosis can affect osteoclasts through the PKC-pathway, which is also probably the mechanism behind the effects of the AAPs (Pereverzev et al., 2008). Moreover, aging has been shown to cause problems in gap junction assembly and function (Genetos, Zhou, Li, & Donahue, 2012), which is of importance in studying the age-related bone diseases. In conclusion, GJC might be the future therapeutic target when developing new treatments for cardiac as well as bone disorders, but further studies are required for revealing the complex control mechanisms occurring in the target tissues.

### 6.4 MSCs as the possible mediators of the differences in the osteoclastogenic capacities of bone marrow and peripheral blood monocytes

#### 6.4.1 Osteoclastogenesis from bone marrow and peripheral blood is differentially regulated with osteoclast growth factors

Since differences were observed in the effects of GJC modulation on osteoclastogenesis depending on the culture conditions and with the possible contribution of MSCs, we characterized the role of the MSCs in osteoclastogenesis in more detail. The studies were made with the human monocyte cultures derived from bone marrow and peripheral blood due to the differences in the presence of MSCs in these sources. Few studies have compared osteoclastogenesis from these
sources (Quinn et al., 1998; Takahashi et al., 1989) albeit both are generally utilized in monocyte isolation for *in vitro* studies.

In the first culture protocol differentiation was induced with RANKL and M-CSF, which have been shown to be sufficient for generation of osteoclasts from the monocytic precursors (Quinn et al., 1998; Yasuda et al., 1998). With this protocol (RM) both bone marrow and peripheral blood derived monocytes differentiated into osteoclasts with no differences in bone resorption activity, secretion of TRACP isoforms or the expression of osteoclast-specific genes *CR* and *NFATc1*. The only difference between the sources was that more osteoclasts formed in the bone marrow derived cultures. The results show that RANKL and M-CSF are sufficient for inducing osteoclastogenesis from both bone marrow and peripheral blood monocytes, and that the larger population of MSCs in the bone marrow does not have a significant effect on the differentiation with this culture protocol.

In the following studies osteoclastogenesis induced with RANKL and M-CSF was further stimulated with TGF-β and dexamethasone (protocol RMTD). With this culture protocol differences emerged in osteoclastogenesis from the two sources. The peripheral blood derived osteoclasts had more nuclei, expressed and secreted higher levels of both TRACP 5a and 5b along with higher expression of *CR* and *NFATc1*, although the differences in expression of these osteoclast-specific genes did not reach statistical significance. The results indicate that the peripheral blood derived monocytes may have an increased potential for differentiation into osteoclasts. This is supported by the theory of the circulating QOPs which have already undergone the initial differentiation phases towards osteoclasts (Muto et al., 2011). Another supportive view is that the dominant monocyte population in blood, the classical monocytes, are the main osteoclast precursors (Komano et al., 2006; Massey & Flanagan, 1999; Nicholson et al., 2000; K. L. Wong et al., 2011).

Although we observed the increased osteoclastogenic potential in the peripheral blood monocytes with the RMTD protocol, it is possible that with the RM protocol osteoclastogenesis from the bone marrow monocytes proceeds at a faster pace. This is explained by the higher number of multinuclear cells in the bone marrow cultures with the RM protocol, as well as the lower levels of TRACP 5a secreted from the bone marrow cultures with both RM and RMTD protocols. Since TRACP 5a is expressed in higher levels in mononuclear cells, their number in the bone marrow cultures might be lower, indicating that most of the cells susceptible to osteoclastogenesis had started the differentiation towards multinuclear cells. Various timepoints have been described for the initial differentiation stages and the beginning of bone resorption for both bone marrow and peripheral blood derived
monocytes depending on the differentiation protocol used, and most of them suggest that osteoclastogenesis from peripheral blood derived precursors would occur faster (Flanagan & Massey, 2003; Lader, Scopes, Horton, & Flanagan, 2001; Merrild et al., 2015; Möller et al., 2017). Our results seemed to indicate the opposite, but to support this conclusion, further timepoint studies are required.

6.4.2 The complex role of TGF-β in MSC-containing osteoclast cultures

An interesting finding was that although osteoclastogenesis and the expression of osteoclast markers were increased in the peripheral blood-derived cultures, the bone resorption activity in this culture was at the lowest level of all studied groups. The reason behind this could be the aforementioned differential regulation of osteoclastogenesis and bone resorption. Fuller et al. showed that while TGF-β stimulated osteoclastogenesis, it did not have an effect on bone resorption (Fuller et al., 2006). Considering the slightly lower number of multinuclear cells but the higher level of bone resorption in the bone marrow derived cultures, TGF-β might have complex roles affecting the results of this study. Depending on the cell culture type, the concentration of TGF-β and the presence of the stromal cells, TGF-β has been shown to have conflicting roles in osteoclastogenesis (Fuller et al., 2000; Houde et al., 2009; Massey et al., 2001; Murakami et al., 1998; Quinn et al., 2001; Susa et al., 2004; Takai et al., 1998). The MSCs in the bone marrow cultures can produce TGF-β (Kassem, Kveiborg, & Eriksen, 2000) leading to higher amounts of it in these cultures compared to the peripheral blood cultures. Interestingly, Karst et al. showed that in the presence of stromal line ST2 cells a high concentration of TGF-β decreases osteoclastogenesis from bone marrow precursors, whereas in stromal cell-free cultures TGF-β stimulates osteoclastogenesis, and secondly, affects both stromal cells and osteoclast precursors (Karst et al., 2004). Although the authors show the multifunctional effects of TGF-β in bone marrow cultures, of note is the fact that they actually compared two cultures both containing the MSCs, since they did not purify the monocytes of the bone marrow from the co-existing population of MSCs by any means before culturing. Considering the osteoclastogenesis-stimulating effect of TGF-β with a low concentration, Karst et al. showed that in this setting TGF-β increased the RANKL/OPG ratio produced by the stromal cells leading to increased osteoclastogenesis. A similar mechanism might also be behind our results, since the peripheral blood cultures are not completely devoid of the MSCs which could produce the increased RANKL/OPG.
ratio upon treatment with a low TGF-β concentration. On the contrary, the bone marrow cultures in our study probably contained an excess of TGF-β due to the amount produced by the MSCs in addition to the recombinant protein supplemented to the cultures, explaining the lower number of multinuclear cells formed. In addition to TGF-β, the MSCs can produce several other osteoclastogenesis-inhibitory molecules contributing to the net effect observed.

6.4.3 The disparity between osteoclasts and macrophage polykaryons and the role of MSCs in their differentiation

Another possible explanation for the higher levels of TRACP 5a secreted from the peripheral blood cultures is that the cultures contain more macrophages, since they are, along with dendritic cells, the main TRACP 5a secreting cells (Janckila et al., 2002; Janckila et al., 2005). Susa et al. (Susa et al., 2004) suggested that when only RANKL, M-CSF and dexamethasone are used for differentiation, the resulting TRACP-positive cells have a low resorption activity and therefore might be macrophage polykaryons rather than osteoclasts. Lader et al. showed that osteoclasts generated in the absence of stromal cells maintain the expression of monocyte-macrophage associated antigens CD11c and CD18, whereas in osteoclasts cultured with the stromal cells the expression of these molecules ceases when bone resorption begins (Lader et al., 2001). In their study, both the osteoclasts in the stromal cell-free culture as well as in the stromal cell-rich culture were bone resorptive cells expressing several osteoclast markers. This shows that although the cells in the stromal cell-free culture remained to express some macrophage-related genes, at least some of them can be considered as real osteoclasts. However, the authors remark that osteoclastogenesis studies without the presence of stromal cells do not reflect the situation in vivo, and might generate possibly erroneous data about the bone cell functions.

The possible presence of the macrophage-type cells in our peripheral blood cultures is also supported by a study by ten Harkel et al., where the authors show that the foreign body giant cells (FBGC) generated from human monocytes with IL-4 and IL-13 showed several similarities to osteoclasts (ten Harkel et al., 2015). For instance, the FBGCs expressed several osteoclastogenic genes, had actin rings and sealing zones along with the capacity to dissolve mineral from hydroxyapatite coated plates. Although the cells were not able to resorb bone, this study shows that the osteoclasts and FBGCs share some similarities, and we cannot exclude the possibility that some of the multinuclear cells in the peripheral blood cultures in
our study were macrophages or FBGCs instead of osteoclasts. According to our findings of the higher number of nuclei in the peripheral blood derived osteoclasts, ten Harkel et al. showed that the FBGCs were larger and had more nuclei compared to osteoclasts. This was also noted in the study of the different osteoclast types, where Sprangers et al. showed that the non-classical monocytes differentiated into multinuclear cells with several nuclei but without bone resorption ability (Sprangers et al., 2016). Due to the lack of the resorptive activity, the authors suggest that the non-classical monocytes may be the precursors for the FBGCs.

As discussed, it seems that osteoclastogenesis from bone marrow and peripheral blood is affected by several regulatory mechanisms and that different growth factors can have distinct effects on osteoclastogenesis from these sources. One aspect requiring further studies is the concentration of the growth factors used in these osteoclastogenesis assays. We initially chose to use the culture protocols optimized for the generation of osteoclasts from the different sources, and therefore the concentrations of RANKL and M-CSF are different between the two protocols. Since the concentrations of both RANKL and M-CSF were higher in the protocol containing also the addition of dexamethasone and TGF-β, we cannot confirm whether the effects are due to these additional growth factors or the increased concentrations of RANKL and M-CSF. Although RANKL has been shown to increase bone resorption dose-dependently (Burgess et al., 1999; Lacey et al., 1998; J. Li et al., 2000), various concentrations are used in osteoclastogenesis assays. Further, some studies have shown that the resorptive activity of mouse osteoclasts is saturated at 10-20 ng/ml concentration and is not increased even with the addition of 100 ng/ml or 500 ng/ml RANKL (Lacey et al., 1998; J. Li et al., 2000). Therefore, our 20 ng/ml and 50 ng/ml concentrations are in the acceptable range concerning osteoclastogenesis, and probably not the main cause behind the effects. A thorough timepoint and concentration curve experiment to determine the effects of RANKL and other growth factors on osteoclastogenesis and bone resorption is currently lacking from the literature, and would further explain the process of osteoclastogenesis from the bone marrow and peripheral blood monocytes.

6.5 Future aspects

In this study we have shown that GJC occurs during osteoclastogenesis and that it can be modulated with either inhibitors or stimulators. The MSCs seem to have a role in the effects of GJC modulation as well as in the overall regulation of osteoclastogenesis. However, we did not study in detail the mechanisms of how the
AAPs act on bone cells. This is of importance if these compounds are to be used to treat bone disorders. Secondly, if they are used for cardiac disorders, their side-effects in other organs should be investigated thoroughly.

To further analyze the roles of the MSCs in osteoclastogenesis, it would be interesting to characterize these cells in regard to their osteoblastic or stem cell properties. Our bone marrow cultures contained the MSCs in high quantities, although the bone marrow sample was first cultured in plastic, where the mesenchymal stem cells should adhere. Therefore, it is possible that the non-adherent MSCs in our cultures represent a subpopulation of the stromal cells with certain osteoclastogenesis regulating properties. Characterization of these cells in regard to the whole stromal cell population of the bone marrow would probably provide an explanation for the complex effects they had in this study.

Concerning the differing monocyte pools in bone marrow and peripheral blood, it would be interesting to analyze further the differences in osteoclastogenesis from these sources. For instance, the aforementioned timepoint and concentration curve study of RANKL stimulation could uncover the questions of the time required for the differentiation along with the starting point of bone resorption between the cell types. The newly developed live visualization techniques for cell cultures might offer beneficial approaches for studying these processes.

Characterization of the monocyte types in regard to their CD expression profile would give answers on what types of monocytes are present in the cultures after the Ficoll-Paque isolation. On the contrary, monocytes can also be isolated from the umbilical cord blood of newborn infants, and although some osteoclastogenesis studies have been made with these cells, their osteoclastogenic potential has not been compared to that of the other monocyte types.
7 Conclusions

GJC between bone cells is one of the regulatory mechanisms in bone homeostasis, but the importance of GJC in osteoclastogenesis is not well known. The specific GJC modulating peptides, AAPs, have shown promising effects in cardiac tissue, but the outcome of GJC modulation in bone has not been broadly studied. Osteoclastogenesis is regulated by complex signaling pathways between osteoclast precursors and other bone cells. Therefore, the aim of this study was to characterize the effects of GJC modulation on osteoclastogenesis as well as the contribution of the MSCs in the process. Based on the results and discussion presented in this study, the following conclusions were made:

1. GJC is one of the cell fusion mechanisms in osteoclastogenesis from both human and mouse monocytic precursors. However, GJC is not indispensable in cell fusion, since other compensatory mechanisms can be utilized in case of inhibited GJC.

2. During osteoclastogenesis, GJC can be stimulated specifically with several AAPs. The AAPs possibly increase Cx43 expression in the fusing monocytes and improve the open probability of the gap junctions in bone cells.

3. The effects of GJC stimulation with AAPs depend on the culture conditions of the target cells as well as on the presence of the osteoclastogenesis-regulating MSCs. The AAPs can exert their activities directly on the differentiating monocytes or indirectly by activating the MSCs, which produce the osteoclastogenesis-regulatory substances. Further, metabolic stress such as acidosis can alter the effects of AAPs in bone cells.

4. The MSCs participate in regulation of osteoclastogenesis in vitro. This was shown with the differing effects of GJC modulation and acidosis on osteoclastogenesis depending on the presence or absence of the MSCs in the cell culture. The contribution of MSCs to osteoclastogenesis became evident also in peripheral blood cultures showing an increased osteoclastogenic capacity due to the lack of the MSCs, which regulate osteoclastogenesis in bone marrow cultures. In addition, depending on the presence of MSCs in culture, different combinations of osteoclastogenic growth factors can generate multinuclear cells with distinct properties, which should be taken into account in osteoclast in vitro studies.
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