Toll-like receptors (TLRs) and inflammatory bone modeling

Ali Kassem
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SWEDISH NATIONAL GRADUATE SCHOOL IN ODONTOLOGICAL SCIENCE

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In the name of God, the most Gracious, the most merciful

To my family, with love

And in memory of my father

“Are they equal, those who have knowledge and those who not have knowledge?”

Quran, 39:9
يَسْمِعُ اللَّهُ الرَّحْمَنُ الرَّحِيمُ

فَلَهُ يَسْتَوِي الَّذينِ يَعْلَمُونَ وَالَّذينَ لاَ يَعْلَمُونَ

سورة الزمر - الآية ٩

هذا من فضلى ربي

الحَمْدُ للهِ الَّذِي وَقَنَى لِهذَا الإِنجِاز

إِهْدِ لِأَهْلِي وَأْحِيي وَأَعْزَاني... مَعَ إِنَّ الْفَضْلِ وَالْعَزَةِ لِلَّهِ جَمِيعًا
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Bone homeostasis
Bone remodeling
Bone modeling

Regulation of Bone homeostasis
Bone and central nervous system
Bone and peripheral sympathetic nervous system
Bone and hormones
Bone and vitamins
Bone and kidney
Bone and adipose tissue
Bone and gut microbiota
Bone and mechanical stimuli (loading or unloading)
Bone and immune system (Osteoimmunology)
Shared mechanisms by immune system and bone
Regulation of osteoclastogenesis by immune cells
Regulation of immune system by bone

Bone, infection and inflammation

Toll-like receptors (TLRs)
Morphology, position and function of TLRs
Ligands of TLRs
Signaling by TLRs
Regulation of TLR signaling
Co-receptors of TLRs

Toll-like receptors (TLRs) and bone

Aims

Methodological considerations

TLR agonists
Animals
Gene and protein expression analyses
Luciferase reporter gene assay
Isolation of cells
Bone organ cultures
Cell cultures
Enzyme-cytochemistry, enzyme-histochemistry and immunohistochemistry
Flow cytometry
Microcomputed tomography (µCT)
Statistics
Results and discussion

Enhanced bone resorption by TLR activation ex vivo

TLR-induced bone resorption is due to enhanced RANKL/OPG ratio

Activation of TLRs induces bone resorption independent of cytokines and prostaglandins

TLR-activation enhances RANKL/OPG ratio in osteoblasts independently of cytokines and prostaglandin

The observed effects are indeed due to activation of TLR2 and TLR5, respectively

*In vivo* activation of TLR2 and TLR5 results in increased osteoclast formation and significant bone loss

The *in vivo* effect by activation of TLRs is associated with increased RANKL/OPG ratio

Signaling pathway in TLR-mediated RANKL expression

Uncoupled bone modeling by activation of TLRs *in vivo*

Conclusions

Acknowledgement

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References
Abstract

Patients with inflammatory or infectious conditions such as periodontitis, peri-implantitis, osteomyelitis, rheumatoid arthritis, septic arthritis and loosened joint prosthesis display varying severity of destruction in the adjacent bone tissue. Bone loss in inflammatory diseases is considered a consequence of cytokine induced RANKL and subsequent enhanced osteoclast formation. Hence, osteotropic cytokines and their receptors have been suggested to be important for the pathogenesis of inflammation-induced osteolysis. It is, here, suggested that bacterial components, so called “pathogen associated molecular patterns=PAMPs”, may also be involved. Varieties of cells express receptors for PAMPs, including Toll-like receptors (TLRs) which are the first line of defence in the innate immune system. LPS (lipopolysaccharide), fimbria and lipoproteins from pathogenic bacteria such as P. gingivalis, S. aureus are ligands for TLR2 and flagellin from pathogenic flagellated bacteria like S. typhimurium is a ligand for TLR5.

Since the susceptibility to, or the severity of inflammation-associated bone diseases are likely related to differences in the tissue response, and the mechanisms by which PAMPs interact with bone cells are not fully understood, we aimed to elucidate the importance of different TLRs for inflammation induced bone loss by conducting in vitro and in vivo investigations.

Activation of TLR2 and TLR5 in organ cultured mouse parietal bones increased bone resorption in a time- and concentration-dependent manner by a process inhibited by OPG and bisphosphonate, showing the crucial role of RANKL-induced osteoclast formation. In addition, the number of osteoclasts, expression of osteoclastic genes and osteoclastogenic transcription factors were increased. In the bones and in osteoblasts isolated from the bones, TLR2 agonists increased the expression of RANKL without affecting OPG, while TLR5 activation resulted in enhanced RANKL and decreased OPG. Activation of both TLR2 and TLR5 stimulated the expression in both bones and osteoblasts of prostaglandins and pro-inflammatory cytokines, known to stimulate RANKL. By
blocking the cytokines and prostaglandin, we showed that TLR2 and TLR5 induced bone resorption and RANKL expression are independent of these molecules.

Activation of TLR2, but not TLR5, in mouse bone marrow macrophage cultures inhibited RANKL-induced osteoclast formation, an effect not observed in committed pre-osteoclasts.

Local administration in vivo of TLR2 and TLR5 agonists on the top of mouse skull bones enhanced local and systemic osteoclast formation and bone resorption. Using knockout mice, we showed that the effects by LPS from P. gingivalis (used as TLR2 agonist) and flagellins (used as TLR5 agonists) are explicit for TLR2 and TLR5 ex vivo and in vivo, respectively.

These data show that stimulation of TLR2 and TLR5 results in bone resorption in vitro and in vivo mediated by increased RANKL in osteoblasts and thus may be one mechanism for developing inflammatory bone loss.

Interestingly, histological analyses of skull bones of mice treated locally with TLR2 and TLR5 agonists revealed that the bones not only reacted with locally increased osteoclastogenesis (osteoclast formation), but also with locally increased new bone formation. This was observed on both periosteal and endosteal sides of the bones, as well as in the bone marrow compartment. The formation of new bone was seen close to osteoclasts in some parts, but also in other areas, distant from these cells. The response was associated with active, cuboidal osteoblasts, extensive cell proliferation and increased expression of genes coding for bone matrix proteins and osteoblastic transcription factors.

In conclusion, activation of TLR2 and TLR5 in osteoblasts results in bone loss associated with enhanced osteoclast formation and bone resorption, as well as with increased osteoblast differentiation and new bone formation, indicating that inflammation causes bone modeling. The data provide an explanation why LPS from P. gingivalis and flagellin from flagella-expressing bacteria can stimulate bone loss. Since TLR2 and TLR5 can be activated not only by bacterial components, but also by endogenous ligands produced in inflammatory processes, the data also contribute to the understanding of inflammation induced bone loss in autoimmune diseases. Hopefully, these findings will
contribute to the development of treatment strategies for inflammation induced bone loss.
Benvävnaden i käkbenet och i skelettet generellt byggs ständigt om för att byta ut gammalt ben mot nytt. Detta åstadkoms genom att osteoklaster resorberar den benvävnad som behöver ersättas och sedan bildar osteoblaster nytt fräscht ben i motsvarande omfattning i detta område. Inflammatoriska processer som parodontit (tandlossning), periimplantit, lossnandet av ortopediska proteser och artrit (inflammation i leder) förändrar ombyggnaden av ben lokalt så att resorptionsprocessen blir mer omfattande än benbildningen vilket leder till att benmassa förloras. På så sätt minskar mängden alveolärt ben kring lossnande tänder och implantat liksom mängden ben i leder hos patienter med artrit. I detta projekt studerar vi hur en infektiöst utlöst inflammationsprocess kan påverka bencellernas förmåga att resorbera ben och bilda nytt ben.

Vid inflammation bildas, dels av invandrade vita blodkroppar och dels av celler i vävnaden kring ben, en mängd olika signalsubstanser som kan påverka omgivande celler. Många av dessa signalsubstanser är cytokiner, ett slags proteiner som alla celler kan bilda och som på ett hormonliknande sätt påverkar framför allt närliggande celler. Några cytokiner frisätts i stor omfattning till blod, i likhet med vanliga hormoner, och påverkar levern att bilda akutfas-proteiner och hjärnan för att åstadkomma feber. Vissa cytokiner som till exempel IL-1β, IL-6, IL-11, LIF, OSM och TNF-α har förmåga att lokalt aktivera benresorption genom att stimulera bildning av RANKL i osteoblaster, en signalmolekyl som är nödvändig för att enkärniga monocyte skall utvecklas till flerkärniga osteoklaster. Denna process hämmas av osteoprotegerin (OPG), en antagonist till RANKL.

Man har upptäckt att många celler, inte bara kan reagera på signaler från cytokiner och liknande kroppsegna signalsubstanser, utan även på molekyler från bakterier via receptorer på eller inuti celler. Detta receptorsystem kallas Toll-liknande receptorer och har stor betydelse för hur en infektion kan påverka våra vävnader och är en viktig del i vårt medfödda immunförsvar. Toll
upptäcktes av tyska forskare (Nüsslein-Volhard och medarbetare; Nobelpristagare 1995) när de hade muterat en gen hos bananfluga fann de att den påverkade fosterutvecklingen och resulterade i en mycket säregen variant av bananflugan. De tyckte då att det var ”helt fantastiskt” (=Toll på tyska) och därmed fick genen sitt namn. Senare fann man att denna gen också hade betydelse för bananflugans infektionsförsvar och sedan upptäcktes att människa har likande gener vilka kodar för receptorer som kan känna igen strukturer hos bakterier, virus och svampar – så kallade Toll-like receptors (TLRs). Dessa receptorer känner igen molekyler hos mikrober, kroppsegna strukturer och främmande matrial vilket resulterar i en mängd effekter på celler som är involverade i inflammation, till exempel bildning av både pro- och anti-inflammatoriska cytokiner men också en direkt påverkan på benceller.

*Porphyromonas gingivalis* och *Staphylococcus aureus* är två viktiga bakterier som associerats med parodontit respektive septisk artrit. Motila ("simmande") bakterier kännetecknas av flagell som är propellerliknande organeller på ena ändan av bakterien och som är uppbyggd av proteinet flagellin. Dessa bakterier är också involverade i parodontit, periimplantit och reaktiv artrit. *Porphyromonas gingivalis* via LPS (lipopolysackarid) och *Staphylococcus aureus* via PGN (peptidoglykan) i sina cellväggar aktiverar TLR2 medan flagellerade bakterier stimulerar TLR5 med proteinet flagellin.

Vid vissa tillstånd såsom osteoporos (benskörhet) och inflammatoriska autoimmuna sjukdomar som reumatisk artrit producerar kroppen signalmolekyler som också aktiverar Toll-receptorer. Dessa endogena TLR aktivatorer kan bidra till eller förvärra destruktiva processer i närliggande benvävnad.

Vi har studerat betydelsen av TLR2 och TLR5 för processer involverade i vävnadsnedbrytning vid parodontit, septisk artrit och andra infektiösa tillstånd samt vid autoimmuna sjukdomar med hjälp av *in vitro* och *in vivo* experiment. Detta genom att analysera bendegraderingsgrad, osteoklastantal och diverse genuttryck av betydelse för benresorption i organ- och cellodling samt i levande möss.
Sammanfattningsvis: aktivering av Toll-receptorer vid bakteriell infektion leder till ökad bennedbrytning via en direkt effekt på osteoblaster som utsöndrar RANKL och därmed ökad osteoklastbildning och aktivitet. Vi har även observerat en ny och mycket intressant funktion för aktivering av TLR2 och TLR5, vilken resulterar i bennybildning. Under ombyggnad av ben är bennybildning kopplat till bennedbrytning medierat av osteoklaster. Vid TLR2 och TLR5 medierad bennybildning har vi observerat detta fenomen också helt skilt från nedbrytningsområden. Denna avhandling förbättrar förståelsen för Toll-receptorer’s roll vid infektiösa och inflammatoriska bennedbrytande tillstånd och bidra till eventuell utveckling av specifika läkemedel mot dessa sjukdomar. Avhandlingen inspirerar även till fortsatt forskning då vi har funnit att stimulering av Toll-receptorer leder till ökad benbildning.
List of publications

This thesis is based on the following original papers, which will be referred to by their Roman numerals:


III. **Ali Kassem**, Catharina Lindholm and Ulf H. Lerner. Toll-like receptor 2 stimulation on osteoblasts mediates *Staphylococcus aureus*-induced bone resorption and osteoclastogenesis through enhanced RANKL. *Manuscript*.


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Related publication

## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>α-MEM</td>
<td>Alpha minimal essential medium</td>
</tr>
<tr>
<td>Acp5</td>
<td>Gene encoding for TRAP</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase domain-containing protein</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance (statistical method)</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bcl6</td>
<td>B cell lymphoma 6 protein</td>
</tr>
<tr>
<td>BMSCs</td>
<td>Bone marrow stromal cells</td>
</tr>
<tr>
<td>BMM</td>
<td>Bone marrow macrophages</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BRC</td>
<td>Bone remodeling compartment</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone sialoprotein</td>
</tr>
<tr>
<td>(^{45}\text{Ca})\</td>
<td>Radioactive Calcium, ([^{45}\text{Ca}]\text{CaCl}_2)</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Calcr</td>
<td>Gene encoding for calcitonin receptor</td>
</tr>
<tr>
<td>CTR</td>
<td>Calcitonin receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>c-Fms (CSF1R)</td>
<td>Colony stimulating factor 1 receptor</td>
</tr>
<tr>
<td>c-Fos</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>c-Src</td>
<td>Cellular sarc (sarcoma) kinase</td>
</tr>
<tr>
<td>Csf1r</td>
<td>Gene encoding for c-Fms</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element (CRE)-binding protein</td>
</tr>
<tr>
<td>CRP</td>
<td>Carbone reactive protein</td>
</tr>
<tr>
<td>CT-1</td>
<td>Cardiotrophin-1</td>
</tr>
<tr>
<td>Ctsk</td>
<td>Gene encoding for cathepsin K</td>
</tr>
<tr>
<td>CTX</td>
<td>C-terminal telopeptide of collagen type I</td>
</tr>
<tr>
<td>Cx43</td>
<td>Connexin 43</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C motif chemokine</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Danger-associated molecular patterns</td>
</tr>
<tr>
<td>DAP12</td>
<td>DNA-activation protein of 12 kDa</td>
</tr>
<tr>
<td>DC-STAMP</td>
<td>Dendritic cell-specific transmembrane protein</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopf 1</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ERα and β</td>
<td>Estrogen receptor α and β</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FLA-B.s.</td>
<td>Flagellin from <em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>FLA-S.t.</td>
<td>Flagellin from <em>Salmonella typhimurium</em></td>
</tr>
<tr>
<td>FZD</td>
<td>Frizzled</td>
</tr>
<tr>
<td>FSL-1</td>
<td>A synthetic lipoprotein from <em>Mycoplasma salivarium</em></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage stimulating factor</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HKLM</td>
<td>Heat-killed preparation of <em>Listeria monocytogenes</em></td>
</tr>
<tr>
<td>HSCs</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of nuclear factor kB kinase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor-associated kinase</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRP5</td>
<td>Low-density lipoprotein (LDL) receptor-related proteins 5</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeats</td>
</tr>
<tr>
<td>MafB</td>
<td>v-maf musculoaponeurotic fibrosarcoma oncogene family member protein B</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>M-CSF (Csf1)</td>
<td>Colony stimulating factor 1</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MEPE</td>
<td>Matrix extracellular phosphoglycoprotein</td>
</tr>
<tr>
<td>Micro-CT (µ-CT)</td>
<td>Microcomputed tomography</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MuOCL</td>
<td>Multinucleated OCL</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NFATc1</td>
<td>Nuclear factor of activated T cells cytoplasmic 1</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>NIK</td>
<td>NF-κB-inducing kinase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OCL</td>
<td>Osteoclast</td>
</tr>
<tr>
<td>OCN</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>OC-STAMP</td>
<td>Osteoclast-stimulatory transmembrane protein</td>
</tr>
<tr>
<td>OPCs</td>
<td>Osteoclast precursors</td>
</tr>
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<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
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<tr>
<td>OSCAR (Oscar)</td>
<td>Osteoclast-associated immunoglobulin-like receptor</td>
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<td>OSM</td>
<td>Oncostatin M</td>
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<td>Osx</td>
<td>Osterix</td>
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<td>Pam2</td>
<td>Palmitoyl-2-Cys-Ser-(Lys)$_4$</td>
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<tr>
<td>Pam3</td>
<td>Palmitoyl-3-Cys-Ser-(Lys)$_4$</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>Porphyromonas gingivalis</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>Prostaglandin E$_2$</td>
</tr>
<tr>
<td>PHEX</td>
<td>Phosphate-regulating neural endopeptidases on X chromosome</td>
</tr>
<tr>
<td>PI3K-Akt</td>
<td>Phosphoinositide 3-kinase-serine/threonine protein kinase</td>
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<td>PRRs</td>
<td>Pattern recognition receptors</td>
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<td>Ptgs</td>
<td>Gene encoding for cyclooxygenase</td>
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<td>PTH</td>
<td>Parathyroid hormone</td>
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<td>PTHrP</td>
<td>PTH-related protein</td>
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<tr>
<td>q-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor kappa B</td>
</tr>
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<td>RANKL</td>
<td>RANK-ligand</td>
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<tr>
<td>RelA</td>
<td>Reticuloendotheliosis viral oncogene homolog A</td>
</tr>
<tr>
<td>RelB</td>
<td>Reticuloendotheliosis viral oncogene homolog B</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Staphylococcus aureus</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>TAB</td>
<td>TAK1-binding protein</td>
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<td>TAK1</td>
<td>TGF-β-activated kinase</td>
</tr>
<tr>
<td>TANK</td>
<td>TRAF family member-associated NF-κB activator</td>
</tr>
<tr>
<td>TGF-α and β</td>
<td>Transforming growth factor alpha and beta</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>TIRAP</td>
<td>TIR-associated protein</td>
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<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>Tnfsf2</td>
<td>Gene encoding TNF-α</td>
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<tr>
<td>Tnfrsf11a</td>
<td>Gene encoding for RANK</td>
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<tr>
<td>Tnfrsf11b</td>
<td>Gene encoding for OPG</td>
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<td>Tnfsf11</td>
<td>Gene encoding for RANKL</td>
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<tr>
<td>TRAFs</td>
<td>TNF receptor-associated factors</td>
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<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
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<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
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<tr>
<td>TREM2</td>
<td>Triggering receptor expressed in myeloid cells-2</td>
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<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter protein-inducing interferon β</td>
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<tr>
<td>Vitamin-D₃</td>
<td>1α,25(OH)₂-vitamin D₃</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>Wnt</td>
<td>Wingless-related integration site</td>
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Introduction

Diseases and pathological conditions affecting bone

Tooth loss is the ultimate consequence of the inflammatory disease periodontitis. Severe form of periodontitis afflicts approximately 10% of the adult population (1). In approximately 20% of the individuals, where lost teeth is substituted with dental implants, an inflammation driven loss of supportive tissue around the implants is detected. This biological complication is denoted peri-implantitis and the most dramatic complication is loss of the implant. Peri-implantitis afflicts up to 47% of patients with dental implants (2, 3). These chronic inflammatory conditions cause a huge discomfort and economic burden (4, 5). The inflammation is initiated by accumulation of bacterial biofilm (dental plaque) on the teeth or implant surfaces in close proximity to the gingival margin. In gingivitis, when the gingiva (the gum) gets red, swollen and easily bleeding due to the inflammatory process, the tooth supporting tissue remains unaffected and the process is reversible (6) (Figure 1A). This condition is called mucositis when it is located around an implant (Figure 1B). In periodontitis, the inflammatory process destroys the supportive tissues, periodontium (periodontal connective tissue and alveolar ridge (jawbone)), in an irreversible manner (6) (Figure 1C). In addition to dysbiotic (unfavorably changed to pathogenic) microbial biofilm, genetics, systemic diseases and inflammatory and environmental factors, especially smoking, contribute to the development and progression of periodontal diseases (7-10). Despite the heavy microbial load on the teeth and chronic inflammation in the gums due to poor oral hygiene, some individuals are resistant to periodontitis while others are highly susceptible (8, 11). Elevated levels of C-reactive protein (CRP) and leukocyte number in serum of patients with progressive periodontitis indicate that periodontal inflammation can also influence the systemic condition (8, 12).

Introducing dental hygiene programs has significantly reduced the prevalence of mild periodontitis in the last four decades, but has failed to change the
prevalence of the severe form of periodontitis, which persists at approximately 10% globally (13). Unfortunately, the increasing replacement of lost teeth with dental implants has not totally solved the problem, since it has become evident that some patients are also susceptible to inflammation-induced implant loosening, peri-implantitis (14) (Figure 1D). Interestingly, patients with history of periodontitis are more often afflicted with peri-implantitis (15, 16). Enormous efforts and extensive research have been made to identify the bacteria causing periodontitis and peri-implantitis, but no single bacteria species could be pointed out as the villain. However, more than 700 bacterial species have been associated with the diseases (17-20), of which *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* and *Treponema denticola* (a flagellated bacteria (21)) are important pathogens (22) (Figure 2A-C). *P. gingivalis*, which possesses many virulence factors (23, 24), is the keystone regulator pathogen in the dysbiotic microbial community (biofilm) that initiates the periodontal disease (8). The sensitivity of the host’s immune system to recognize and react to pathogens is a very important element in the pathogenesis (development of disease) of periodontitis (25). Lipopolysaccharide (LPS; known also as Endotoxin), the main component of the cell wall of Gram-negative bacteria, is one of most important and key virulence factors. *P. gingivalis’* LPS differs from other bacteria’s LPS due to modifications in the Lipid A moiety, and can activate different receptors (8, 26). These differences in LPS of *P. gingivalis* are due to culture conditions and environmental factors. Moreover, *P. gingivalis* has the ability to evade the immune system by inhibiting the inflammatory cytokines IL-12, IL-23 and IL-27 in macrophages through activation of complement factor C5a by gingipains (C5a-convertase-like enzymes) production, and subsequently inducing periodontal attachment loss (i.e. bone destruction) (27-29).

In order to combat these infectious agents, the body is equipped, *inter alia*, with Toll-like receptors that initiate the immune system to respond.
Rheumatoid arthritis (RA) is an inflammatory autoimmune disease that affects the joints and destroys the cartilage and surrounding bone (Figure 3A). Epidemiological studies have linked RA with periodontitis, showing that RA patients exhibit higher prevalence of periodontitis and the severity of both diseases is related to each other (30-33). Treatment of periodontitis results in beneficial effects on RA disease activity, leading to fewer tender and swollen joints, decreased CRP and decreased pain (34). Pathogenic bacteria \emph{P. gingivalis} is the only bacteria known to utilize peptidyl-arginine deiminase (PPAD) enzyme to produce citrullinated proteins (35). The immune system attacks citrullinated proteins and produce anti-citrullinated protein antibodies (ACPAs), leading to
autoimmune diseases. ACPAs are highly specific markers for RA and play a key role in the etiology of the disease (36). These findings support the hypothesis that periodontitis is an inducer of ACPA formation and contributes to the debut of RA.

Figure 2. Bacterial morphology. (A) The pigmentation of *P. gingivalis* colonies on blood agar. (B) Electron micrograph of *P. gingivalis*, the strain ATCC 33277. (C) Flagellated bacteria. (D) The pigmentation of *S. aureus*. (Nakayama K, *J of Periodontal Res*, 2015)

**Septic arthritis** is a serious, rapid and destructive condition that affects the joints and erodes the surrounding bone (Figure 3B), and that is caused by purulent bacterial invasion. The most common and frequent bacterial species associated with septic arthritis in adults and children is *Staphylococcus aureus* (*S. aureus*) (37, 38) (Figure 2D). Septic arthritis has a global prevalence of up to 0.01 %, but patients with RA or orthopedic implants (artificial joints) are more susceptible (39, 40). Septic arthritis is characterized by pain, swelling, redness and warmth in the infected joint. Septic spread of *S. aureus*, not only affects bone locally in the joints bust also has an osteoporotic (bone degrading) effect on the skeleton (41).

**Loosened joint prosthesis** is a growing costly issue for the society because of increased joint-replacement therapies nowadays (42). The loss of orthopedic implants in association with post-operative infections can be prevented by optimal hygiene routines and treatment with antibiotics. However, the sterile inflammatory loss of these prosthesis (Figure 3C) is harder to manage since it is clinically asymptomatic until total failure of the implants (43). The reason for loosened joint prosthesis is peri-prosthetic osteolysis induced by wear particles such as polyethylene and polymethylmethacrylate. These foreign materials
promote osteoclast formation and bone resorption by inducing inflammatory mediators (43) initiated by Toll-like receptors (44).

**Osteomyelitis** is a condition, often caused by *S. aureus*, in which the bacteria infect and colonize the bone marrow. Osteomyelitis, especially in children, is a morbid disease characterized by aggressive suppurative bone destruction in the acute phase and focal or cortical/periosteal bone thickening (sclerosis) in the chronic phase (40, 45-47) (**Figure 3D**).

All the above-mentioned diseases are inflammatory conditions, mediated by the immune system, affecting bone. The usual outcome of these conditions is pathological bone loss. The susceptibility and/or the severity of these conditions vary among individuals/patients, influenced by genetic elements.

**Figure 3.** Showing bone tissue affected by inflammation or infection. (A) Rheumatoid arthritis. (C) Septic arthritis. (B) Loosened prosthesis. (D) Osteomyelitis. (250)
**Bone biology**

- **Bone tissue**

**Bone composition, morphology and different types of bone**

Bone tissue consists of cells and mineralized extracellular matrix. The matrix is composed by an organic (*i.e.* osteoid) and an inorganic component (*i.e.* mineral crystals). The organic part is mostly made up of collagen type I fibers (90% of the total protein). The remaining 10% are non-collagenous proteins that include glycoproteins (*e.g.*, bone sialoprotein (BSP), osteonectin, fibronectin, thrombospondin and vitronectin), γ-carboxylated proteins (*e.g.*, osteocalcin) and proteoglycans (*e.g.*, aggrecan, decorin and versican). Moreover, exogenously derived proteins like α2-HS (Heremans-Schmid)-glycoprotein and serum albumin, and osteoblast-produced growth factors such as bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs) and transforming growth factor-beta (TGF-β) are also present in bone matrix. The inorganic part is constituted of mineral crystals, mainly hydroxyapatite [Ca_{10} (PO_{4})_{6} (OH)_{2}], which comprise approximately 65% of the tissue weight. Acid phosphate, carbonate and magnesium crystals, in small amounts, are also present in the inorganic portion of the matrix. The composition and organization of the matrix’s micro- and macrostructure provides the bone tissue its special biomechanical properties as strength, rigidity, stiffness and lightness (48, 49).

Bone texture is divided into compact or **cortical** bone, which makes up 80% of total bone mass, and the remaining spongy or **trabecular** bone (also known as cancellous bone). The complex architecture of bone is built up by porous inner core of trabecular bone surrounded by dense and solid outer shell of cortical bone. Trabecular bone is composed of interconnected Honeycomb-like networks of trabecula (alike rods and plates), which is about 50-300 μm in diameter. Both cortical and trabecular bone are constituted by osteons units that in trabecular bone, are semilunar and called packets, but in cortical bone, are cylindrical and called Haversian systems. Haversian systems are formed by concentric (circular) lamella with a central canal (Haversian canal) through which blood vessels extends in parallel to the long axis of the bone. Cortical bone contains canals in
the transvers axis that are called Volkmann’s canals and harbor blood vessels and nerves. Canaliculi interconnect lamellar layers that embed cells in between (in the lacuna). Haversian systems, which range between 10-500 μm in diameter, form a branching network within the cortical bone and are surrounded by circumferential lamella. Lamella lining between osteon are called interstitial lamella. Cortical bone has an inner and outer cell- and blood vessels-rich connective tissue membrane called endosteum and periosteum, respectively. Woven bone is produced during primary bone formation and lacks lamellar structures due to irregularly arranged collagen fibrils. Woven bone is weak and usually seen in high bone turnover conditions (e.g., Paget's disease) or the high bone formation due to early fluoride treatment. (48-50) (Figure 4).

Figure 4. Schematic illustration and histological picture of bone microanatomy. (a) Cortical and trabecular bone. (b) Cross-section view of osteon with osteocytes. (c) Histological photograph of cortical bone.
Functions and importance of bone

The bone tissue in the skeleton provides the body structural support (shape) and protection for internal organs and enables movement through attachment of muscles, joints, ligaments and tendons. Bones house the blood cells production in the bone marrow, contributing to hematopoiesis (51), and contribute to maintenance of acid-base balance and mineral homeostasis by acting as a reservoir of calcium and phosphate (48). Moreover, bones enable the energy supply by providing support mechanism for teeth for mastication and contribute to energy balance in an endocrine manner (secretion internally into the systemic circulation) (52, 53). In addition, bone tissue participates in the maintenance of immune system and hormonal balance (54, 55) and regulates male fertility (56).

- Cells of bone tissue

Four different cell types are present in bone tissue, including osteoblasts, osteocytes, lining cells and osteoclasts. Osteoprogenitor cells, known as bone marrow stromal cells (BMSCs), are pluripotent mesenchymal cells that can differentiate into osteoblasts. Stromal cells are usually regarded to be derived from mesenchymal stem cells located in bone marrow in near proximity to bone surface, but have also been found in other tissues such as adipose tissue, amniotic fluid, dental pulp, muscle, skin and umbilical cord. These mesenchymal cells possess the ability to differentiate into adipocytes, chondrocytes, fibroblasts, myoblasts and osteoblasts dependent on the received signal from the microenvironment, which, among other things, could explain the ectopic ossification in non-skeletal tissues (50, 57).
**Osteoblasts: Morphology, formation and functions**

Active osteoblasts are mononucleated cells that generally are round or cuboid in shape and line up on bone surfaces. They have typical secretory characteristics with strong basophilic cytoplasm, granules containing pro-collagen and proteoglycans, well-developed rough endoplasmic reticulum with dilated cisterna, large Golgi complex and voluminous nucleolus. In addition, these cells exhibit cytoplasmic processes, which extend into the osteoid matrix and connect to osteocyte processes. Osteoblasts represent only 4-6% of total resident cells in bone tissue and abundantly express alkaline phosphatase (ALP) in the plasma membrane, which is used as a characteristic marker for osteoblasts. Mature osteoblasts lack the ability of proliferation (growth via cell division) (50, 58) (Figure 5).

![Figure 5](image)

**Figure 5.** Histological section of intramembranous bone formation with active and inactive osteoblasts.
Osteoblasts arise from the osteoblastic-linage differentiated from mesenchymal stromal cells (MSCs) under the influence of different signal molecules such as (59, 60):

- Growth factors (e.g., TGF-β, fibroblast growth factors (FGFs) and IGF)
- Bone morphogenic proteins (BMPs)
- Cytokines (e.g., leukemia inhibitory factor (LIF), interleukin 6 (IL-6), IL-11 and oncostatin M (OSM))
- Hormones (e.g., parathyroid hormone (PTH) and 1,25(OH)2-vitamin D3)
- Cell-cell interaction and cell-extracellular matrix (ECM) interaction
- Mechanical loading
- Local factors, including Wnt (Wingless-related integration site) proteins
- MicroRNAs (miRNAs)

MSCs differentiation to osteoblasts requires a specific set of gene expression (61). MSCs, under the control of Wnt10b and BMPs, are induced toward chondro-osteoprogenitors, which differentiate to committed osteoprogenitors and further to pre-osteoblasts and later on to mature osteoblasts (59). Runt-related transcription factor 2 (Runx2; formerly called Cbfa1) is a master regulator of osteoblast differentiation, especially in the early development of committed osteoprogenitor from chondro-osteoprogenitors (61). Runx2-deficient mice lack osteoblasts and develop a skeleton made of cartilage exclusively, where chondrocytes fail to undergo hypertrophy (62). However, Runx2 transgenic mice (overexpressing) develop osteopenia, due to decreased mature osteoblast number, which indicates the role of Runx2 in negative control of terminal differentiation of osteoblasts (63). Thus, Runx2 controls osteoblast differentiation and bone formation differently at several levels. Runx2 induction is regulated by various stimuli like, BMPs, FGF, PTH, TGFs, and mechanical loading (64). Osterix (Osx) is another important transcription factor required specifically for osteoblast differentiation (i.e. unlike Runx2, is not required for chondrocyte hypertrophy) (65). Mice lacking Osx fail to form bone due to
absence of osteoblast differentiation (66). Osx forms a complex with nuclear factor of activated of T-cells cytoplasmic 1 (NFATc1) and acts downstream Runx2, regulating the expression collagen type I (61, 65). BMPs and IGF-1 stimulate Osx expression in MSCs. Distal-less homeobox 5 (Dlx5) is an osteogenic transcription factor expressed in chondro-osteoprogenitors stimulated by BMPs, which is also activated during fracture healing. Dlx5 modulates Runx2-activity and is involved in the induction of bone sialoprotein (BSP) and osteocalcin (OCN) (59). In addition, Sox4 (SRY-related HMG-box4) is an important transcription factor that stimulates proliferation, differentiation and function of osteoblasts in a Runx2-independent way upstream Osx (67). Moreover, bagpipe homeobox homolog 1 (Bapx1), C/EBPs (CCAAT/enhancer-binding proteins), Smads, ATF4 (activating transcription factor 4), Satb2 (especial AT-rich sequence-binding protein 2), transcriptional coactivator TAZ and AP1 (activator protein 1)-family transcription factors play significant role in osteoblast differentiation and maturation by regulating Runx2 (59, 65). In addition to Runx2 induction, activation of Wnt system promotes further commitment of chondro-osteoprogenitor to pre-osteoblasts. Several other factors interfere and inhibit osteoblast differentiation like (65):

- P53
- Proliferation-activated receptor γ (PPARγ)
- Hoxa-2, Msx1, Msx2 (homeobox proteins)
- Twists (basic helix-loop-helix (bHLH)-containing transcription factors)
- Schnurri (zinc finger proteins)
- MEF (myeloid Elf-1-like factor)
- STAT1 (signal transducers and activators of transcription 1)
- Sox9, Sox8
- Nrf2 (nuclear factor (erythroid-derived 2)-like 2)
- YAP (Yes-associated protein).

Wnt proteins (Wnt1, 3a, 4, 5a, 7b and 10b among 19 secreted proteins) promotes osteoblast differentiation and activation (68). In humans, loss-of-function mutations in LRP5 (a Wnt co-receptor) leads to osteoporosis-
pseudoglioma syndrome (69) and gain-of-function mutation to high bone mass syndrome (70, 71). Moreover, osteoblast differentiation and function is regulated by several miRNAs, acting as both stimulators and inhibitors (65). Osteoblast differentiation from MSCs can also be initiated by activated monocytes/macrophages through OSM signaling (72). When pre-osteoblasts transform to large cuboidal differentiated osteoblasts, they cease to proliferate, start to express ALP and secrete bone matrix proteins like collagen type I, osteopontin (OPN) and BSP. Ultimately, upon maturation and activation, osteoblasts express genes involved in mineralization of bone matrix as well as OCN, which is a marker of mature osteoblasts with hormonal activity (59) (Figure 6).

Figure 6. Schematic illustration describing osteoblastogenesis and the regulation by transcription factors.

The most significant osteoblast function is to produce bone, a process where these cells initially synthesize the organic bone matrix (osteoid) and then facilitate its mineralization. Osteoblasts deposit collagen type I fibers in an organized way and secret the non-collagen proteins like BSP, OPN, OCN and osteonectin (59). This is followed by the mineralization process in two steps, hydroxyapatite crystals formation in dispatched matrix vesicles and release and growth of mineral crystals on and in-between collagen fibrils. Formation of so-called critical nucleus (or nucleation cone) is crucial for the first stable crystal
establishment. Subsequent elongation of hydroxyapatite crystals involves several enzymes (49, 73). Osteoblasts exert another vital function by managing the differentiation and regulation of other cells like MSCs, hematopoietic stem cells (HSCs) (74), B-lymphocytes (75) and osteoclasts (bone-destroying cells) (76).

Osteoblast signaling

Osteoblasts utilize several different intracellular signaling pathways. For instance, PTH via its receptor (PTHr) initiates G-protein signaling cascades that enhance the expression of Runx2 (only for a short period) and other transcription factors promoting osteoblast differentiation, proliferation/survival and function (77). TGF-β and BMPs (BMP2, 4-7) induce osteogenesis by binding to their receptors through canonical and non-canonical signaling pathways (78). Canonical Smad-dependent system enhances Runx2 expression by formation of transcription factor complexes comprised of R-Smads with co-Smad and Smad4. The non-canonical induction of Runx2 is mediated through TAK1 (TGF-β activated kinase 1)-p38 MAPK (mitogen-activated protein kinase) cascade (78). BMP2 can also induce Runx2 via PI3K-Akt2 (phosphoinositide 3-kinase – serine/threonine protein kinase) signaling (79) (Figure 7A,B). Several Wnts bind to FZD-LRP5/6 receptor complex [Frizzled (FZD; seven-loops-transmembrane G protein-coupled receptors (GPCRs) and low-density lipoprotein (LDL) receptor-related proteins (LRPs)]. This complex activates intracellular signals that inhibit GSK-3β (glycogen synthase kinase-3 β) activity, leading to prevention of β-catenin phosphorylation. Non-phosphorylated β-catenin is stable and after accumulation in the cytoplasm, upon reaching a specific concentration, translocates into the nucleus where it regulates transcription of Wnt-target genes. In the absence of Wnt, GSK-3β phosphorylates β-catenin, thus promoting its ubiquitination and subsequent prevention of lymphoid-enhancer-binding factor (LEF)/T-cell-specific transcription factors (TCFs) activation. Wnt/β-catenin signaling pathway is the canonical pathway, but Wnts can also signal via non-canonical pathways. Dickkopf 1 (DKK1) and sclerostin inhibit Wnt/β-catenin signaling pathway by binding to LRP receptors, preventing the LRP/FZR integration (68). Wnt3a
signals in synergy with heparin to engage PI3-Akt2 pathway and in parallel with β-catenin signaling, alter Runx2 in osteoblasts (80). Upon connection of osteoblasts to ECM by integrins (e.g., α2β1, α4β1, α5β1), they elicit signals via PI3K-Akt2 and/or ERK (extracellular signal-related kinase)-MAPK cascades (60).

Figure 7 A. Schematic illustration of TGF-β signaling in osteoblasts (81).
Recently, it was discovered that osteoblast-secreted osteocalcin has an important role in energy metabolism by stimulating pancreatic β cells proliferation and insulin secretion, as well as in fertility by stimulating the maturation of Leydig cells and their testosterone production. Therefore, osteoblasts promote bone formation further by enhancing the release of anabolic testosterone (56).

**Figure 7 B.** Schematic illustration of BMP signaling in osteoblasts (81).
Osteoblasts’ fate, after bone formation phase, varies as they undergo apoptosis (programmed cell death), become inactive osteoblasts (bone lining cells) or differentiate into osteocytes. Approximately, 60-80% of active osteoblasts undergo apoptosis after bone formation (82).

**Osteocytes: Morphology, formation and functions**

Osteocytes have a spider-shaped appearance with long filopodial processes (microspikes) encased in lacuna (small space). They are distributed evenly throughout the mineralized matrix and are interconnected; building a network that enables metabolical and electrical exchange (ions and small molecules). Osteocytes, through their dendritic processes located in tiny canals called canaliculi, connect to cells lining the bone surface, to the bone marrow and blood vessels (83, 84). Osteocytes represent 95% of cells present in bone tissue (excluding bone marrow) and arise from only 10% of destined osteoblasts (83, 84) (Figure 8).

**Figure 8.** Osteocytes in electron microscope image (Courtesy of Kevin Mackenzie).
Osteocytes are non-proliferative, terminally differentiated cells, originated from osteoblasts. The designated mature and active osteoblasts decelerate matrix production and are progressively embedded in it. At this stage, the cell becomes an osteoblastic osteocyte or type I pre-osteocyte, which advances towards an osteoid osteocyte (type II pre-osteocyte), a type III pre-osteocyte, a young osteocyte and finally an old osteocyte (82, 83, 85). This transformation takes approximately three days and is characterized by striking changes, from polygonal morphology to a flat cell with several extending dendrites that reaches out to the mineralizing front, vascular space or bone surface in a polarized manner. Osteocytes are motile (within the lacuna) and able to extend or retract their processes (82, 85). Differentiation of osteocytes and formation of osteocytic processes requires collagen and other matrix proteins cleavage, since mice lacking MT1-MMP (matrix type 1-metalloproteinase) exhibit osteocytes with shorter and fewer dendritic processes (86, 87). In osteocytes, ALP, BSP, OCN and collagen type I are downregulated or limited, while other molecules are highly expressed like (82, 84, 85):

- DMP1 (dentine matrix protein 1; involved in regulation of mineralization and differentiation of osteocytes)
- casein kinase
- FGF23
- MEPE (matrix extracellular phosphoglycoprotein)
- PHEX (phosphate-regulating neural endopeptidases on X chromosome)
- E11 (membrane-bound protein E11)
- CD44 (Cluster of differentiation; receptor for hyaluronic acid, osteopontin, collagens and MMPs)
- SOST (encoding for Sclerostin).

FGF23, PHEX and MEMPE are involved in phosphate homeostasis. Unlike osteoblasts, osteocytes are enriched in proteins associated with hypoxia (e.g., ORP150; oxygen-regulated protein) and cytoskeletal function (e.g., E11, destrin, Cap-G (macrophage-capping protein) and Cdc42 (cell division control protein).
SOST encodes for sclerostin, which is a specific marker for mature osteocytes. Since sclerostin is an inhibitor of Wnt/β-catenin pathway, by blocking LRP5, loss-of-function mutation in SOST causes high bone mass in human (88). Mechanical loading and PTH decrease the expression of sclerostin, resulting in enhanced bone formation. This is believed to be the mechanism for the anabolic effect of mechanic loading (84). Despite the immense progress in identification of osteocyte markers, it remains unclear which factor is the key regulator of osteocyte differentiation. The final fate of osteocytes is degradation during osteoclastic bone resorption (82).

Early recognized functions assigned to osteocytes are mechanosensation and mechanotranduction. Osteocytes are the key mechanotransducers in bone, sensing the mechanical strain composed of three types of stimuli: deformation of bone matrix, fluid flow and shear stress. The sensory organelles of osteocytes are the cell body, primary cilium and dendritic processes (via integrins) (84, 89). Osteocytes respond to mechanical loading by biochemical signals affecting bone formation and/or resorption. Integrins transduce the stimuli through connecting ECM to either the cytoskeleton or connexin 43 (Cx43) and thereby open hemichannels that regulate the release of prostaglandin E₂ (PGE₂) (84, 89). Wnt/β-catenin pathway is one important signaling pathway activated by mechanical strain (84, 89). In addition, Wnt/β-catenin pathway plays an important role in gap junction function, communication, and viability (prevents glucocorticoid-induced apoptosis by enhancing Cx43) of osteocytes (84). Mechanical loading activates other signaling pathways that cross talk with Wnt/β-catenin pathway (84, 89). Moreover, RANKL expression increases in osteocytes due to unloading (90) and osteocyte-specific RANKL-knockout mice are resistant to the unloading-induced bone loss (91). However, osteocyte viability has a very important role in maintenance of bone homeostasis and damage repair (84). Osteocytes exert endocrine functions and participate in the regulation of phosphate homeostasis, through DMP1, PHEX, MEPE and FGF23 pathways by regulating phosphate reabsorption in the kidneys. DMP1, PHEX and MEPE proteins are also involved in bone mineralization (84, 89). Osteocytes participate in calcium homeostasis by removing and subsequently replacing the
perilacunar (space surrounding the osteocyte) mineralized matrix (called perilacunar remodeling) (84, 89). During lactation, osteocytes induce perilacunar bone remodeling by enhanced PTHrP (PTH-related protein) via PTH receptor (92) (see further below, section: Bone remodeling and hormones). In addition, osteocytes can influence muscle cells proliferation and growth by secreting factors like PGE2, MCP-1, sclerostin and Wnt3a (93). Osteocytes are the central orchestrator of bone formation and resorption by integrating the mechanical and hormonal signals and by affecting both osteoblasts and osteoclasts (84, 89). Osteocytes are able to recruit MSCs to the fracture site by secreting osteopontin (94). They also regulate osteoblast differentiation and activation by releasing stimulators, including nitric oxide (NO), IGF-1, PGE2 and Adenosine triphosphate (ATP), and inhibitors such as sclerostin, DKK1 and secreted frizzled-related protein 1(SFRP1) (84). Moreover, osteocytes express macrophage-colony stimulating factor (M-CSF) and RANKL to enhance, and NO, osteoprotegerin (OPG) and TGF-β to inhibit osteoclast formation and activity (95). Osteocytes are the major RANKL-producing cells in physiological bone remodeling. Apoptosis of neighboring osteocytes, inflammation, excessive PTH and unloading promote, while sex steroids inhibits RANKL production by osteocytes (84, 89, 95).

However, despite all the essential functions attributed to osteocytes, acellular bone (without osteocytes) in many fishes exhibit the same activities as bone with osteocytes (i.e. response to mechanical stimuli and remodeling). So, is there a redundancy or alternative ways? (96).

**Bone lining cells: Morphology, formation and functions**

Bone lining cells descent from osteoblasts and directly appose to the inactive (no modeling or remodeling) bone surface. They are flat elongated quiescent cells with thin nucleus and few cytoplasmic organelles, but include mitochondria, microfilaments, free ribosomes, and sparsely rough endoplasmic reticulum. These cells connect to, and communicate with each other through gap junctions and extended processes (64, 97) (Figure 9).
Bone lining cells protect the bone surface and play an important role in regulation of local mineral homeostasis, flow of ions between interstitial fluid and bone and hematopoiesis (97, 99). These cells act as a reservoir of “determined” osteogenic precursors that, under appropriate conditions, can differentiate into reverse cells and active osteoblasts (97, 100). Moreover, bone lining cells have an important role in bone resorption and remodeling. They digest non-mineralized collagen protruding from the bone surface and retract to give access to osteoclasts. Then, these lining cells differentiate into reversal cells that colonize the resorption track, forming the canopy of bone remodeling compartment (BRC), synthesizing bone and attract osteoblasts and pre-osteoblasts into the BRC (99-101). Bone lining cells are believed to rescue bone remodeling in pycnodysostosis patients and cathepsin K–deficient mice, in which their resorptive ability depends on the activity of MMPs and cysteine proteinases (99).
Osteoclast morphology

Osteoclasts, in general, are giant multinucleated (have several nuclei) cells formed from fusion of several macrophage cells originated from HSCs. The size of mature osteoclasts varies and usually is 20-100 micron in diameter, but osteoclasts can also be small and mononucleated (102) (Figure 10). The several nuclei (up to 50) vary in shape, from round to irregular, and each nuclei might have different transcriptional activity (103). Osteoclasts contain several enzyme-containing lysosomes, endosomes, rough and smooth endoplasmic reticulum and multiple Golgi complexes. They also house many elongated mitochondria, to provide the high levels of energy expenditure required for their activity (102). Mature and active osteoclasts are polarized and typically circular or half-spherical in shape (like a hat) supported by actin fibers and rings (102).

Osteoclasts have an apical membrane facing the bone surface, basolateral membrane towards the top of the cell facing the vascular stream and a functional secretory domain on the top. The apical membrane has a ruffled border and consists of hyper-specialized zones (102). The localization of the organelles in osteoclasts is peculiar and functionally oriented. Generally, nuclei are surrounded by endoplasmic reticulum and Golgi apparatuses, located in the vascular side of the cell (the top) while mitochondria and other vesicles are mostly located in the apical side of the cell (the base) facing the bone (Figure 11). Microfilaments that support the morphology of the cell stretch in the periphery while microtubules verge from their center nearby the nuclei toward the periphery, facilitating the membrane trafficking to the ruffled border. Intermediate filaments, constituted of vimentin, form a basket around the nuclei and extend toward the cell edge. These filaments may contribute to the cell’s shape and sensing deformity (102). The apical membrane comprises sealing zone and ruffled border, which is divided into outer fusion zone and inner uptake zone. The sealing zone contains clear zone and sealing membrane that is enriched in special and dynamic adhesions, the podosomes (Figure 11). Podosomes, characterized by fast turnover and rapid regulation, allow the dynamic attachment of osteoclasts to the bone matrix. They are made of actin microfilaments and proteins like actin-binding, adhesion, adapter and signaling proteins along with tyrosine kinases and integrin receptors. Podosomes start
building as single foot-like molecules that eventually cluster, move to the periphery and form a podosomal belt that anchors and give rise to actin ring. In addition to podosomal belt, osteoclasts bridge the microfilaments to the ECM by integrins, especially α2β1, αvβ3 and αvβ5 (102, 104). Osteoclasts are characterized by expression of a series of markers, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, CTR (calcitonin receptor), CAII (carbonic anhydrase II), Atp6i (the a3 subunit of the vacuolar [H+]−ATPase), ClC-7 (chloride channel 7), and OSTM1 (osteopetrosis-associated transmembrane protein 1). The best markers visualizing osteoclasts are TRAP and cathepsin K (102, 105).

**Figure 10.** Osteoclast morphology. (A) Electron microscope image of an osteoclast. (B) Osteoclasts of various sizes and nuclei number with resorption track, stained for TRAP. (C) Histological section of tooth eruption with many osteoclasts, stained for TRAP. (Courtesy of Tim Arnett).
Figure 11. Illustration of osteoclast morphology. (A) Actin microfilament showing peripheral podosomal belt. (B) Progression of podosomes in osteoclasts. (C) Osteoclast morphology with different cellular compartments. G: Golgi apparatus; N: nucleus; M: mitochondrion; RER: rough endoplasmic reticulum; L: lysosome (102).

**Osteoclast differentiation**

Osteoclast differentiation is highly complex and occurs through a series of steps involving commitment of HSCs into the myeloid pool and further to monocyte/macrophage lineage, proliferation and differentiation of osteoclast precursors, fusion of mononuclear cells and polarization and maturation (104, 106). HSCs, under the stimulation of stem cell factor (SCF), IL-3 and IL-6, commit to multipotent common myeloid progenitors (CMPs) (107). This process is controlled by the transcription factor PU.1, which belongs to the family of ETS (E26 transformation-specific) transcription factors, recognizing a purine-rich sequence 5’-GGAA-3’. PU.1 together with a heterodimeric complex of MITF (microphthalmia-associated transcription factor; basic-loop-helix-leucine zipper) and TFE3 (transcription factor binding to IGHM enhancer 3) drive c-Fms expression (also known as Csf1r, colony stimulating factor 1 receptor, gene encoding for the tyrosine kinase receptor for M-CSF) (104, 107, 108). In addition, PU.1, in conjunction with MITF, up-regulate RANK expression (receptor activator of NF-κB). During osteoclastogenesis, corepressors of MITF and PU.1 association, including Eos (a zinc finger protein; an Ikaros transcription factors family member), decrease allowing the amplification of PU.1/MITF complex (104, 107, 109). GM-CSF (Granulocyte/macrophage-colony stimulating factor) promotes CMPs differentiation into granulocyte/macrophage progenitors (GMPs) that are further induced into osteoclast precursors (OCPs) of
monocyte/macrophage lineage by M-CSF. Expression of CD11b (known also as integrin \( \alpha_M \)) and CD14 is the marker of immediate GMPs that after commitment (i.e. OPCs) start to express Mac-1 (macrophage-1 antigen or integrin \( \alpha_M\beta_2 \)), c-Src (cellular Src kinase; "sarc", as it is short for sarcoma, is a proto-oncogene encoding a tyrosine kinase), c-Fms and RANK. M-CSF (also known as CSF-1 (colony stimulating factor 1)) is a cytokine that promote the proliferation, differentiation and survival of precursor cells of monocytes/macrophages lineage as well as RANK expression (106-108). M-CSF signaling through its receptor c-Fms activates MITF, which bind to Bcl-2 promoter and regulate the expression of anti-apoptotic protein Bcl-2 (B-cell lymphoma 2) (104, 106, 107). Two different isoforms of MITF are expressed by macrophages and osteoclasts, MITF-A and MITF-E, of which MITF-E expression increases during the differentiation of osteoclasts (104, 110). MITF interacts also with PU.1 inducing the transcription of osteoclast specific genes, including Ctsk (encoding for cathepsin k) and Acp5 (encoding for TRAP) (104, 106, 110) (Figure 12). M-CSF was initially identified as hematopoietic growth factor that induced macrophage colony formation from bone marrow progenitors and regulated the female reproductive tract cells (111). Several cell types like vascular endothelial cells, gingival and synovial fibroblasts, lymphocytes, BMSCs, osteoblasts and osteocytes, express M-CSF, of which BMSCs, osteoblasts and osteocytes are the major producer of M-CSF in the bone microenvironment (108). Considering the concept of bone remodeling within the BRC (i.e. osteoblasts differentiate after osteoclast differentiation/activation), osteocytes are likely the main supplier of M-CSF in the BRC (108). M-CSF, due to differential splicing, is expressed as either predominant secreted proteoglycan, secreted glycoprotein or cell-surface membrane-bound protein (111).
Figure 12. Schematic illustration of osteoclast differentiation.

**M-CSF and osteoclast mobilization, proliferation, survival and differentiation**

M-CSF binds to and activates c-Fms (cellular homolog of the feline transforming virus v-Fms), which is a transmembrane receptor with a cytoplasmic tyrosine kinase domain expressed only on cells of macrophage lineage (108). c-Fms activation takes place through either dimerization of the receptor monomers or conformational change of a pre-assembled dimer. Activation of c-Fms via conformational change enhances the activity of the tyrosine kinase domain by trans-phosphorylating specific tyrosine residues, which then serve as high-affinity binding sites for SH2 (Src homology region 2) domain-containing signaling molecules (108). The cytoplasmic domain contains eight tyrosine (Y) residues that can be activated, of which Y544, Y559, Y697, Y721, Y807 and Y921 are functionally involved in the regulation of OPCs proliferation/survival. Supposedly, phospho-Y807 causes the conformational change of the c-Fms intracellular domain. Phospho-Y559 interacts with c-Src activating PI3K-Akt pathway, but also promotes receptor ubiquitination by recruiting c-Cbl complex (Casitas B-lineage lymphoma) (108). This ubiquitination changes the configuration of the tyrosine kinase domain resulting in amplification of phosphorylation and full receptor activation. Phospho-Y721 activates PI3K-Akt pathway directly while phospho-Y697 and phospho-Y974 activate the Ras/Raf/MEK/ERK cascades via Grb2/Sos complex (growth factor receptor-
bound protein 2/son of sevenless protein). Thus, phospho-(Y559, Y697, Y721 and Y974) enhance OCPs proliferation and phospho-(Y559, Y697, Y721 and Y974), most likely, prolong OCPs survival through the ERK and PI3K-Akt pathways (108) (Figure 13). M-CSF also potentiates RANKL-induced bone resorption in mature osteoclasts independent of survival. Furthermore, M-CSF contributes to bone resorption by participating in the rearrangement of cytoskeleton by co-signaling with αvβ3 integrin in osteoclasts via shared c-Src-initiated signaling complex (108). M-CSF changes the conformation of αvβ3 to its high affinity state, which in turn activates Rac signaling. Thus, M-CSF participates in cytoskeleton reorganization of osteoclasts by inside-out activation of the integrin (108). Moreover, M-CSF can directly affect cytoskeleton organization through forming a multimeric signaling protein complex [comprised of DAP12 (DNAX-activation protein of 12 kDa) and the Syk (non-receptor tyrosine kinase) and Grb-2(growth-factor-receptor-bound protein 2)], binding to Y559/697/721 of c-Fms cytoplasmic domain, activating PI3K-Akt signaling pathway (108). Interestingly, M-CSF elicits a negative auto-feedback that control and balance the survival and proliferation of OCPs (112). This mechanism is wielded by SHIP (Src homology 2-containing inositol-5-phosphatase), which is recruited by Shc to the intracellular domain of c-Fms. SHIP dephosphorylates PIP3 and thereby inhibits PI3-Akt signaling pathway (113). M-CSF induced OCPs proliferation can be inhibited by Krox20 (also known as EGR2; early growth response protein 2). Krox20 interferes with c-Fms expression and ERK signaling pathway (114).
IL-34, a cytokine with no sequence homology or structure similarity to M-CSF, also binds to, and signals through c-Fms. IL-34 binds to different Fms domain, exhibits a different bioactivity and activates signal pathways with different kinetics/strength. IL-34 activates the ERK 1/2 and Akt pathways and enhances the adhesion and proliferation of OCPs (108, 115). In addition, a variety of cells like gingival fibroblasts, lymphocytes and osteoblasts express IL-34, promoting osteoclastogenesis (116, 117). Moreover, IL-34 can entirely substitute for M-CSF in RANKL-induced osteoclast formation in vitro and promotes osteoclastogenesis in vivo (118). However, considering the spatiotemporal (place and time) differences in expression of M-CSF and IL-34, and the complete lack of osteoclasts in osteopetrotic mice (op/op) and toothless rats (tl/tl), we can conclude that IL-34 cannot substitute for M-CSF in normal bone remodeling and it only might be important for osteoclastogenesis in certain pathological conditions (e.g., RA). Besides IL-34, other growth factors like FTL-3L, HGF, PlGF (placental growth factor) and VEGF (vascular endothelial growth factor)
can substitute for M-CSF to stimulate osteoclast formation, which may also have a role in osteoclastogenesis in bone disorders (108, 118).

Mobilization and recruitment of osteoclast precursors (OCPs), from either the bone marrow or blood stream (capillaries) to the bone matrix, requires a variety of molecules that include S1P (sphingosine-1-phosphate; known also as lysosphingolipid) (119) and cytokines such as (107):

- GM-CSF
- SCF
- IL-3, IL-7, IL-12
- FTL-3L (Fms-like tyrosine kinase 3 receptor ligand)

and chemokines like:

- IL-8
- MCP-1 and 3 (monocyte chemoattractant protein-1),
- MIP-1α (macrophage inflammatory protein-1α), MIP-4
- RANTES (regulated upon activation normal T-cell expressed and secreted)
- SDF-1 (stromal cell-derived factor-1; also known as CXCL12 (C-X-C motif chemokine 12))

Either mesenchymal cells (e.g., BMSCs, osteoblasts, lining cells or osteocytes) or lymphoid cells (e.g., B-cells and T-cells) release these factors. Moreover, systemic factors and hormones play a significant role in osteoclastogenesis (107).

In summary, M-CSF is one of two essential osteoclastogenic factors along with chemoattractant and stimulatory molecules that facilitate differentiation and mobilization of OCPs to the designated site, where further commitment to pre-osteoclasts and fusion to mature osteoclasts is carried out.

The next step in osteoclastogenesis is the crucial RANKL (RANK-Ligand) signaling through its receptor RANK, which generates committed pre-osteoclasts and further on, promotes the fusion to multinucleated osteoclasts. RANKL is the other essential cytokine in osteoclastogenesis, although RANKL-RANK signaling is not exclusive for osteoclastogenesis.
**RANK, RANKL and OPG**

RANK, also known TRANCE-R (TNF-related activation-induced cytokine receptor), ODAR (osteoclast differentiation and activation receptor) or CD265, is a type I transmembrane glycoprotein with four extracellular cysteine-rich pseudo-repeats, and belongs to TNF receptor superfamily (like OPG). It is 616 amino acid long and expressed in few cell types like OPCs, mature osteoclasts, dendritic cells and some cancer cells as well as in mammary glands and brain (106, 120, 121). Since RANK's large intracellular domain lacks intrinsic enzymatic activity (kinase activity to phosphorylate signaling molecules), upon ligation by RANKL, the receptor homotrimerizes and recruits adapter molecules (e.g., TRAFs, tumor necrosis factor receptor-associated factors) to its cytoplasmic tail in order to transduce signals (106, 108). Five TRAF proteins (TRAF 1, 2, 3, 5 and 6) interact with RANK, which has three functional (in osteoclastogenesis) TRAF-binding motifs: Motif 1 (PFQEP$_{369-373}$), Motif 2 (PVQEET$_{560-562}$) and Motif 3 (PVQEQQ$_{604-609}$) (106, 108). TRAFs are adapter proteins that transduce signals by recruiting protein kinases. Motif 1 binds TRAF6 and Motif 2 recruits TRAF3 while Motif 3 interacts with TRAF 2 or TRAF 5. IVVY$_{535-538}$ is another RANK motif that mediates macrophage commitment to osteoclast-lineage, but also contributes to osteoclast differentiation (108) (Figure 14).

![Figure 14. Schematic illustration of RANK singling and TRAF-binding sites (108).](Image)
RANKL [also known as ODF (osteoclast differentiation factor) OPGL (osteoprotegerin ligand), and TRANCE (TNF-related activation-induced cytokine)] is a 40-45 kDa type II transmembrane protein that contains a membrane-anchoring domain, a connecting shaft and a receptor-binding ectodomain and belongs to the TNF (tumor necrosis factor) superfamily (106, 108, 121). It forms stable homotrimers (three non-covalently bound identical macromolecules) and exist as three isoforms, namely RANKL1, RANKL2 and RANKL3. It can be truncated to shorter intracellular domain (RANKL2) or cleaved and released as soluble protein (sRANKL or RANKL3) (108). The proteolytic cleavage of RANKL is mediated by ADAM10, 17 or 19 (a disintegrin and metalloproteinase domain-containing protein) or MMP3, 7 or 14 (108). RANKL is expressed in a variety of tissues such as bone, brain, heart, joints, lung, lymph nodes, mammary gland, periodontal tissue, placenta, skeletal muscle, skin, stomach and thyroid gland (108, 120). RANKL is the exclusive ligand for its cognate receptor RANK, playing a crucial role in bone metabolism, lymph node formation and activation in the immune system, mammary gland development during pregnancy, cancer, fever and female body temperature regulation (108, 120, 122). In addition, RANKL might have an effect on cardiovascular system (123).

OPG [osteoprotegerin; also known as OCIF (osteoclastogenesis inhibitory factor), FDCR-1 (follicular dendritic cell-derived receptor-1) and TR1] is a secreted soluble glycoprotein that forms 110 kDa disulfide-linked homodimers and belongs to TNF receptor super family (120, 124). OPG is expressed in the brain, heart, liver, lung, intestines kidney, placenta, skeletal muscle, skin, stomach and testicle (120, 124). OPG is the natural decoy receptor for RANKL, controlling its activity (inhibits RANKL from binding to its RANK). The expression ratio of RANKL and OPG is important for osteoclast formation in physiological and pathological conditions (120, 121, 124).
Terminal differentiation of osteoclasts and RANKL-RANK signaling

In unstimulated OCPs, TRAF 2 and TRAF 3 form a complex with cIAP1/2 (the cellular inhibitor of apoptosis 1 and 2), resulting in ubiquitination and degradation of NIK (NF-κB-inducing kinase; regulator of non-canonical NF-κB pathway), which limits osteoclast formation. RANKL signal induces TRAF3 degradation, allowing the accumulation of NIK and subsequent induction of osteoclast formation (125). TRAF6 activates MAPKs pathways by forming a signaling complex with TAB1 (TAK1-binding protein), TAB2 and TAK1 that recruit Gab2 (Grb2-associated binding protein 2) resulting in activation of ERKs, IKKs (inhibitor of nuclear factor kB kinase), JNK (Jun N terminal kinase), and P38 pathways as well as canonical NF-κB (108, 126). This signaling complex activates p38 via MKK6 (MAPK kinase 6) resulting in activation of STAT1 and MITF, and subsequent induction of the genes encoding cathepsin K, TRAP and OSCAR (osteoclast-associated receptor) (107, 121). ERK1/2 (extracellular signal-related kinase 1 and 2) are also activated by MEK1/2 (MAPK-ERK kinase 1 and 2) in conjunction with PKC-β (protein kinase C member) leading to ELK (a ETS domain-containing transcription factor) activation and consecutive gene expression involved in formation of osteoclasts (107). ERK phosphorylation negatively regulates the p38 activation and vice versa. Thus, probably the balance between P38 and ERK pathways controls osteoclastogenesis (107, 125). TRAF6 also engages JNK pathway by its upstream kinase MKK7 leading to activation of c-Jun and subsequently c-Fos and AP-1 (activator protein-1) transcription factors, which up-regulate NFATc2. In addition, TRAF6-TAB1/2-TAK1-Gab2 complex induces NF-κB pathway through IKKs, enhancing the expression of cFos and AP-1. NF-κB dependent c-Fos and NFATc2 cooperatively induce NFATc1 expression, which is the key factor and master regulator of terminal osteoclast differentiation (107, 121, 125, 126). NFATc1, trough enhanced c-Fos, is also induced at a later stage by CaMK4 (Ca^{2+}/calmodulin-dependent kinases type 4) and CREB transcription factor (cAMP (cyclic adenosine monophosphate) response element (CRE)-binding protein) dependent manner (126, 127). In synergy with AP1-c-Fos complex, Ca^{2+}/calcineurin mediate the activation (i.e. dephosphorylation and translocation into nucleus) of NFATc1. Then, NFATc1 in cooperation with other transcription factors, like AP-1 complex,
PU.1, and MITF regulates a number of osteoclast-specific genes encoding for Atp6v0d2 (the d2 isoform of the vacuolar ATPase Vo domain), β3 integrin, CTR, DC-STAMP (dendritic cell-specific transmembrane protein), cathepsin K and OSCAR (107, 126, 127). NFATc1 up-regulates its own expression and the AP-1-containing c-Fos complexation with NFATc1, together with calcineurin-mediated continuously activated calcium signaling, is necessary for this self-autoamplification (107, 126, 127). NFATc1 is not only a positive regulator of osteoclast formation, but also functions as a transcriptional repressor of anti-osteoclastogenic genes, such as IRF8 (interferon regulatory factor 8), Bcl6 (B cell lymphoma 6), and MafB (v-maf musculoaponeurotic fibrosarcoma oncogene family member protein B), by inducing BLIMP1 (B lymphocyte-induced maturation protein-1) (107, 126, 127). MafB, a basic leucine-zipper transcription factor important for hematopoiesis (128), interferes with DNA-binding of c-Fos, MITF and NFATc1 (128, 129). Bcl6 binds to NFATc1, cathepsin K and DC-STAMP promoters suppressing their expression, but it dissociates upon RANKL stimulation (129). IRF8, a transcription factor important for commitment of myeloid cells to monocyte lineage, binds to interferon-stimulated response element and arrest the differentiation of OCPs at early stage (129). Ids (inhibitors of differentiation/DNA-binding) binds to MITF and prevent its DNA-binding activity (129). Moreover, TRAF6 binds c-Src to recruit Cbl and activate Gab2 that induce PI3K-Akt signaling pathway, leading of up-regulation of NFATc1 (126, 127). TRAF1 and TRAF5 also contribute to the process of osteoclast differentiation via pathways that result in activation of NF-κB and p38, but the exact mechanism is elusive (125) (Figure 15).

Osteoclastogenesis is cooperatively induced by M-CSF, RANKL and its costimulatory factor, immunoglobulin-like receptor. The binding of M-CSF to its receptor, c-Fms, activates the proliferation, cytoskeletal reorganization, survival of osteoclast precursor cells of the monocyte/macrophage lineage, and induces RANK expression. RANKL-RANK binding results in the recruitment of TRAF6, which activates NF-κB and JNK-c-Jun. RANKL also stimulates the induction of a component of AP-1, c-Fos, which is dependent on NF-κB and CREB. AP-1 contributes to the robust induction of NFATc1, which is based on an autoamplifying mechanism that is effected through persistent calcium signal-mediated activation of NFATc1 (NFATc1 binds to NFAT-binding sites on its own promoter, constituting a positive feedback loop). The immunoglobulin-like
receptors (IgLR; OSCAR, PIR-A, TREM-2, and SIRPβ1) associate with FcRγ and DAP12, both of which contain ITAM. ITAM signaling functions as a costimulatory signal for RANK, which is essential for Syk activation, phosphorylation of PLCγ, and subsequent activation of calcium signaling. The tyrosine kinases Btk and Tec, activated by RANK, also promote phosphorylation of PLCγ, thus linking the RANK and ITAM pathways. In the nucleus, NFATc1 cooperates with other transcription factors, such as AP-1, PU.1, CREB and MITF to induce various osteoclast-specific genes. On the other hand, NFATc1 activity is negatively regulated by other transcription factors, such as IRF-8, Bcl6 and MafB during osteoclastogenesis. Blimp1 is induced by RANKL through NFATc1 and functions as a transcriptional repressor of anti-osteoclastogenic genes. Thus, NFATc1 choreographs the determination of cell fate in the osteoclast lineage by inducing the repression of negative regulators as well as through its effect on positive regulators.

**Figure 15.** Schematic illustration of RANK-RANKL signaling (126).
Since RANK is not the only TRAF binding receptor, but the only one responsible for osteoclastogenesis, the specific mechanisms underlying this exclusiveness remain to be identified. However, some cofactors like Gab2, EGFR (epidermal growth factor receptor), FHL2 (four- and-a-half LIM domain 2), Lyn, CYLD (tumor suppressor cylindromatosis) and TNFAIP3 (NF-κB negative regulator A20) deubiquitinases and TANK (TRAF family member-associated NF-κB activator) have been suggested to modulate RANK signaling in osteoclasts, where CYLD, TNFAIP3 and TANK do so negatively (121, 126, 127, 130).

**Costimulatory signaling in osteoclast differentiation**

Osteoclast formation requires costimulatory signal from a receptor complex that is activated by RANK signaling. This synergistic signaling is necessary, but not sufficient (i.e. it cannot induce osteoclast formation by itself) (126, 127). The cooperative receptor complex comprises of cell membrane-anchored IgLR (immunoglobulin-like receptors), including OSCAR, PIR-A (paired immunoglobulin-like receptor), SIRPβ1 (signal-regulatory protein β1) and TREM2 (triggering receptor expressed in myeloid cells-2), and intracellular adaptor molecules like DAP12 and FcRγ (fragment crystallizable receptor γ subunit) (126, 127). DAP12 and FcRγ are ITAM (immunoreceptor tyrosine-based activation motif)-binding proteins that associate with IgLR, which lacks intracytoplasmic tail and therefore rely on FcRγ and DAP12 for intracellular signaling (126, 127). Upon activation, RANK phosphorylates ITAM in DAP12 and FcRγ, recruiting Syk (cytoplasmic protein-tyrosine kinase), Btc (Bruton’s tyrosine kinase) and Tec protein kinases, which activate PLCγ (phospholipase C γ) leading to calcium mobilization and activation of calcineurin (a calcium-dependent phosphatase). This activity enhances NFATc1 and CREB activities (126, 127) (Figure 15). Moreover, RANK regulates calcium oscillation by RGS10 (regulator of G-protein signaling 10) and controls calcium flux by a TMEM64/SERCA2 (transmembrane protein 64/ sarcoplasmic endoplasmic reticulum Ca²⁺ ATPase 2) dependent mechanism, further promoting CREB and NFATc1 activity (131, 132). The DNA-binding molecule RBP-J (recombination signal binding protein for immunoglobulin kappa J region or recombinant recognition
sequence binding protein at the Jκ site; also known as CSL and CBF1; the major activator of Notch signaling) suppresses ITAM-mediated co-stimulatory signaling by inhibiting the expression and function of PLCγ2 and its subsequent activation of downstream calcium signaling. This results in suppression of NFATc1, BLIMP1, and c-Fos, which prevents downregulation of IRF8 (133).

**NF-κB signaling in osteoclast differentiation**

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells or nuclear factor-kappa B) is a heterodimeric transcription factor that can be constituted of five different subunits, namely p50 (NF-κB1), p52 (NF-κB2), p65 (also known as RelA; reticuloendotheliosis viral oncogene homolog-A), RelB and c-Rel (134, 135). All subunits have an N-terminal Rel-domain for dimerization and four sequence-specific DNA binding domains, but only RelA, RelB, and c-Rel have TAD (C-terminal transcription activation domain) (135). Because p50 and p52 lack TADs, they must interact with other subunits to regulate gene transcription (135). RelA and c-Rel preferentially bind to p50 while RelB associates with p52 (135). In the inactive form, NF-κB dimers are bound to IkBs (inhibitors of NF-κB activity; e.g., IκBα, IκBβ, and IκBε) in the cytoplasm. p100 (precursor of p50) functions also as an inhibitor by binding to RelB (135). IκBα predominantly binds p50-RelA while IκBε preferentially regulates RelA-RelA and c-Rel-RelA. IκBβ also binds to p50-RelA (135). Upon activation, NF-κB complex translocates into the nucleus and starts the expression of various genes. Dependent on type of stimuli, NF-κB is activated in two distinct pathways, resulting in different biological effects (134-136). The classical or canonical pathway is immediate and rapid, but transient and short due a potent negative feedback loop generated by upregulation of IκBα, being one of the earliest transcriptional targets of NF-κB (134, 135). The alternative or non-canonical pathway is delayed since it is dependent on TRAF3 degradation (134, 135). Upon activation of canonical pathway, IKK (IκB kinase; a trimeric complex of the catalytic subunits, IKKa and IKKβ, and a regulatory subunit, IKKy also known as NEMO: NF-κB essential modulator) phosphorylates IκBα, resulting in its polyubiquitination and degradation and thereby activation by heterodimerization and translocation of p50-p65 dimer (135). The non-canonical pathway is activated when TRAF3 is
detached from TRAF2-TRAF3-cIAP1/2 (cellular inhibitor of apoptosis proteins 1 and 2) complex, releasing the activated NIK, which phosphorylates IKKα. Active IKKα then phosphorylates p100, leading to processing of p100 to p52 and thereby allowing the dimerization of p52-RelB and subsequent translocation (135). Both canonical and non-canonical NF-κB signaling pathways are necessary for RANKL induced osteoclast formation (134-136) (Figure 16).

Figure 16. Schematic illustration of canonical and non-canonical NF-κB signaling in osteoclastogenesis (135). RANKL and TNF induce canonical signaling by recruiting TRAF6 and TRAF2/5, respectively, to their receptors to activate a complex consisting of IKK-α, IKK-β and IKK-γ (NEMO), which induces phosphorylation and degradation of IκB-α and the release of p65/p50 heterodimers, which translocate to the nucleus. This induces expression of c-Fos and NFATc1 as well as the inhibitory κB protein, NF-κB p100. In unstimulated cells, p100 binds to RelB to prevent its translocation to the nucleus. RANKL induces the ubiquitination and lysosomal degradation of TRAF3 through TRAF2/cIAP1/2 releasing NIK to activate (phosphorylate) IKK-α, which leads to proteasomal processing of p100 to p52; RelB:p52 heterodimers then go to the nucleus to induce target gene expression. TNF does not degrade TRAF3, and thus NIK is degraded, leading to the accumulation of p100 in the cytoplasm of osteoclast precursors to limit their differentiation.
Recently, it was reported that the non-canonical NF-κB pathway is indispensable and modulates RANKL-induced osteoclast formation and mitochondrial biogenesis (formation) by a NFATc1 independent mechanism (137). Moreover, the non-canonical NF-κB pathway in osteoclastogenesis is regulated negatively by NLRP12 (nucleotide-binding leucine-rich repeat and pyrin domain-containing receptor 12) (138).

**Osteoclast fusion**

After differentiation, OCPs fuse and form multinuclear giant osteoclasts. Cell-cell fusion takes place under the command of RANKL (139). Osteoclast fusion has three steps: First, recruitment and alignment of adhesion molecules on the opposing neighboring cells by membrane lipid rafts. Second, interaction of these adhesion molecules with each other and subsequent induction of actin rearrangement and intracellular signal transduction. During this step, the opposing membranes get closer. Third, actin polymerization facilitates the direct contact of opposing membranes pushing the phospholipid bilayers to merge and form a fusion pore. Tensions created by actin polymerization cause the expansion of fusion pores (140). Syncytin-1 is involved in the fusion of the plasma membranes lipid bilayers (141). Molecules suggested to participate in osteoclast fusion are categorized in RANKL-independent molecules like CD44, CD47, Connexin 43, E-cadherin and TREM2, and RANKL-dependent molecules, such as CD9, CD81, ATP6v0d2, DC-STAMP and OC-STAMP (139). CD47, a cell surface integrin-associated protein, binds to SIRPα (signal regulatory protein α) and arbitrates cell-cell recognition prior to cell-cell fusion (139). CD44, a type I transmembrane glycoprotein, connects the cell surface to several ECM proteins. When activated, CD44 intracellular domain is cleaved and translocated into nucleus, activating NF-κB pathway. CD44 contributes to organization of cytoskeletal actin by linking to podosome cores (139, 142). In addition, upon contact, extracellular domains of CD47, SIRPα and CD44 undergo a cleavage to help the cells get closer (139, 142). Migration and formation of protrusion in pre-fusion osteoclasts is initiated by PI(3,4,5)P3 and/or PI(3,4)P2 (phosphatidylinositol 3,4,5-trisphosphate) production on the membrane (143). Blocking CD47 results in larger osteoclasts with more nuclei. In contrast,
blocking Cx43 leads to formation of smaller osteoclasts with fewer nuclei, often binucleated (142). However, the lack of CD47 impairs stromal cell mediated osteoclast formation in vitro and results in an osteopenic bone phenotype in vivo (144, 145).

DC-STAMP and OC-STAMP (osteoclast stimulatory transmembrane protein), seven-transmembrane proteins, are induced by RANKL and essential for osteoclast fusion, as ATP6vod2 is required for optimal cell-cell fusion (139, 146). NFATc1 directly regulates expression of DC-STAMP, OC-STAMP and ATP6vod2 (139, 146). In OC, DC-STAMP is a dimeric receptor with an intracellular C-terminus while dendritic cells’ DC-STAMP is located on the endoplasmic reticulum (ER) (140). Moreover, fusogenic molecules like ADAM12 (also called Meltrin-α), ADAM8, E-cadherin, ITAM1 (intercellular adhesion molecule-1) and LFA1 (leukocyte function-associated antigen-1) and adhesion molecules, including integrins (αv, β1 and β3) are involved in osteoclast fusion and maturation (139, 140). OCPs fuse to form large polykaryons according to basic and evolutionary preserved modalities: An immobile fusion acceptor engulfs a mobile fusion donor, a multinuclear immobile cell increases its nuclei number by taking up a mobile mononuclear cell and a less differentiated pre-osteoclast prefers to merge with a more differentiated pre-osteoclasts or osteoclasts (142). Two types of cell surface contacts precede the act of fusion, broad contact surface or phagocytic cup-like (147). The preference for dissimilarity in differentiation stages between fusion partners might be a way of insuring that fusion only takes place at the right time and in right place on the bone surface instead in the bone marrow (142, 147).

In summary, positive regulators like NFATc1, ITAM and Ca\(^{2+}\) and negative regulators such as TRAF3, Bcl6, IRF8 and MafB balance osteoclastogenesis, induced by M-CSF and RANKL in TRAFs, NF-κB and calcium-signaling dependent ways. In addition, OPC chose fusion partners based on heterogeneity in differentiation status.
Osteoclast function: Polarization and bone resorption

The main osteoclast function is to resorb bone. Osteoclasts are highly specialized cells and equipped with unique tool-kit to achieve the task. Osteoclasts resorb bone by dissolving the mineral crystals of hydroxyapatite and enzymatically cleaving the organic matrix component (collagen fibers and non-collagen proteins) (102, 108). Motile non-polarized (flattened) osteoclasts move by forming and spreading lamellipodia (membrane protrusions) and podosomes (148). Once the resorption site is reached; osteoclasts undergo cytoskeletal reorganization and polarize (148). Osteoclasts attach to bone by sealing zone isolating the resorption compartment, which is opposed to its ruffled border. Ruffled border is the morphological characteristic of resorptive organelle in osteoclasts. Its formation is probably a result of membrane facing the rough and irregular bone surface and a way to increase the area to house proton pumps, H-ATPases, chloride channels and vesicle transportation systems (108, 149). Equipping ruffled border with its functional units is mediated by VT-SNAREs (vesicular and target soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) and Syt VII (synaptotagmin 7) (108, 149). Furthermore, proteins regulating autophagy, including Atg4B, Atg5, Atg7 and LC3 are involved in the formation of ruffled border (108). Osteoclasts adhere to bone by binding to matrix proteins like vitronectin, osteopontin and bone sialoprotein via integrin αvβ3, which exposed after bone lining cells or osteocytes have dissolved the no-mineralized osteoid on the bone surface (108, 150). Activation of αvβ3 induces cytoskeletal organization and polarization of resorptive machinery to the interface with bone. This activation triggers intracellular signaling cascades through phosphorylation of c-Src (108, 149). Cytoskeletal reorganization induced by αvβ3 results in actin ring formation and consequently isolation of resorption compartment (108, 150). Osteoclasts acidify the bone to dissolve the mineral in the resorption compartment by releasing protons (H⁺) via V-H⁺-ATPase (vacuolar-type proton-adenosine triphsphatase) (102, 108). The 2Cl⁻/1H⁺ antiporter (chloride channel type 7) balances the electric charge created by proton transport. The proton pump and chloride channel’s β-subunit (known as OSTM1) are likely located in outer fusion zone of the ruffled border (102, 108). Acidification (pH 4.5) process is also dependent on
the enzymatic activity of carbonic anhydrase type II (CA II) as a source of protons by hydration of CO\(_2\) in H\(_2\)CO\(_3\) as well as on the anion exchanger situated in the basolateral membrane. Anion exchanger manages Cl\(^+\) influx and HCO\(_3^-\) outflow preventing cytoplasmic alkalinization (102, 108).

In order to degrade the organic matrix, osteoclasts release proteolytic lysosomal enzymes. PLEHKM1 (pleckstrin homology domain containing family M member 1) protein and mannose-6p mediate the trafficking of vesicles (102). Acidic hydrolases (proteolytic enzymes), like cathepsin K are transported and released into resorption compartment, where they degrade collagen fibers. Degradation products are transported from the resorption compartment by a process called transcytosis (102, 108). Waste products are endocytosed (vesicular uptake in the uptake zone of ruffled border) and transported through the cell towards the non-resorptive side along a transcytotic vesicular pathway, where they are secreted out from the functional secretory domain (102, 108). Osteoclasts have several lysosomal enzymes, including TRAP and cathepsins. Cathepsin K is a cysteine protease that degrades collagen type I (102, 108). TRAP function is still not fully elucidated, but it is enzymatically cleaved and converted into ATPase by cathepsin or trypsin, recognizing TRIP-1 (TGF-β receptor-interacting protein 1) (102). It is also involved in osteopontin dephosphorylation. MMP-2 is involved in osteoclast development while MMP-9 is involved in osteoclast migration and together with MMP-13 contributes to degradation of collagen type I in addition to cathepsin K (151). MMP-12 participates in the cleavage of the functional domains of bone sialoprotein and osteopontin (102) (Figure 17).
Other osteoclast functions

Osteoclasts have other functions than bone resorption. Osteoclasts respond to hormonal signals and participate in calcium and phosphate homeostasis (102, 152). Osteoclasts participate in orchestrating bone remodeling by cross talking to osteoblasts, regulate osteoblastogenesis and bone formation (102, 152). Osteoclasts participate in regulation of hematopoiesis and maintenance and mobilization of the HSC niche through either providing more space for bone marrow or through metabolism of several important HSC regulatory factors, including CXCL12 (known as SDF-1), GRO (growth regulated oncogene), c-kit ligand (also called SCF) and UDP-glc (uridine diphosphate-glucose) (102, 152). In addition, osteoclasts regulate immune system by exerting effects on T cells (Treg), inducing the release of IL-2, IL-6 and IFNγ (interferon γ), and FoxP3 (forkhead box P3) expression (102, 152). Interestingly, OCPs and osteoclasts, via release of NO, inhibit T cell proliferation (152). Moreover, osteoclasts modulate angiogenesis (blood vessel formation) by producing VEGF during osteoclastogenesis via NF-κB and HIF-1α (hypoxia inducible factor 1α) pathways
and possibly through TGF-β (known to induce VEGF) release from bone matrix during bone resorption (102). Osteoclasts induce angiogenesis by heparinase production, an enzyme that digests heparin sulfate proteoglycans releasing heparin-binding growth factors, including VEGF and bFGF (basic fibroblast growth factor) (102). Other angiogenesis stimulating factors released by osteoclasts are BMP7, HB-EGF (heparin-binding epithelial growth factor), AREG (amphiregulin), EREG (epiregulin) and NRG (neuregulin) (102).

Alternative pathways to RANKL signaling in osteoclastogenesis

Despite the crucial role of RANK-RANKL in both physiological and pathological osteoclast differentiation and formation, evidence exists showing alternative RANKL-independent pathways in osteoclastogenesis. TNF-α induces formation of non-functional osteoclasts in vitro, but simultaneous activation of TRAF6 by IL-1 rescues osteoclast activity in vitro (153). This highlights the essential role of TRAF6 for cytoskeletal reorganization and complete activation of mature osteoclasts. In addition, IL-6 (+ sIL-6R) and IL-11 can induce the formation of mature and active osteoclasts in vitro in a gp-130 dependent, but RANKL-independent manner (154). TGF-β stimulates formation of small authentic osteoclasts (with resorptive activity), independent of RANKL, TNF-α, IL-6 and IL-11 in vitro (155). With TGF-β being abundantly present in the bone matrix, this mechanism could present a possible complementary osteoclast induction pathway to RANKL pathway. SOFAT (secreted osteoclastogenic factor of activated T cells) is a cytokine that induces osteoclast formation in vitro independent of RANKL (156). SOFAT can probably act as exacerbating factor in inflammatory conditions. Furthermore, APRIL (also called TNFSF13; a proliferation inducing ligand), BAFF (also called TNFSF13b; B-cell activating factor), IGF-1/2 and NGF (nerve growth factor) stimulate osteoclast formation and activation in vitro independent of RANKL (157). RANKL independently formed osteoclasts are fewer and exhibit much lower resorptive activity compared to RANKL-induced osteoclasts. These alternative pathways most likely may not replace RANKL, but probably contribute to osteoclastogenesis in certain milieus (153-157).
Osteoclasts and microRNAs

MicroRNAs (miRNA or miR) are single-stranded non-coding RNAs constituted of 18-25 nucleotides that play significant roles in many physiological and pathological processes. They exert both stimulatory and inhibitory effects. Several microRNAs regulate osteoclast formation and/or activity. (Table 1) (158-164).

Table 1. The effect of miRNAs on osteoclastogenesis. N: negative effect; P: positive effect.

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Effect</th>
<th>miRNAs</th>
<th>Effect</th>
<th>miRNAs</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-7b</td>
<td>N</td>
<td>miR-29b</td>
<td>N</td>
<td>miR-141</td>
<td>N</td>
</tr>
<tr>
<td>miR-9</td>
<td>P</td>
<td>miR-29c</td>
<td>P</td>
<td>miR-146a</td>
<td>N</td>
</tr>
<tr>
<td>MiR-16</td>
<td>P</td>
<td>miR-31</td>
<td>P</td>
<td>miR-148a</td>
<td>P</td>
</tr>
<tr>
<td>miR-17</td>
<td>N</td>
<td>miR-34a</td>
<td>N</td>
<td>miR-155</td>
<td>N</td>
</tr>
<tr>
<td>miR-20a</td>
<td>N</td>
<td>miR-124</td>
<td>N</td>
<td>miR-181a</td>
<td>P</td>
</tr>
<tr>
<td>miR-21</td>
<td>P</td>
<td>miR-125a</td>
<td>N</td>
<td>miR-190</td>
<td>N</td>
</tr>
<tr>
<td>miR-26a</td>
<td>N</td>
<td>miR-126-5p</td>
<td>N</td>
<td>miR-210</td>
<td>P</td>
</tr>
<tr>
<td>miR-29a</td>
<td>P</td>
<td>miR-133b</td>
<td>N</td>
<td>miR-214</td>
<td>P</td>
</tr>
<tr>
<td>miR-183</td>
<td>P</td>
<td>miR-223</td>
<td>N</td>
<td>miR-503</td>
<td>N</td>
</tr>
<tr>
<td>miR-218</td>
<td>N</td>
<td>miR-378</td>
<td>P</td>
<td>miR-9718</td>
<td>P</td>
</tr>
<tr>
<td>miR-219</td>
<td>N</td>
<td>miR-422a</td>
<td>P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Osteoclast heterogeneity**

Under embryonic bone development, ossification (bone formation) occurs in two different ways, namely endochondral ossification in long bones and intramembranous ossification in skull- and jawbones (165). This results in different site-specific matrix composition and osteoblastic types (osteoblasts, lining cells and osteocytes), which manifest in different and bone-site-specific bone remodeling times, hormonal responses, neuronal responses and mechanically-induced responses (165). Different populations of osteoclasts also show heterogeneity in size, function and gene expressions (165, 166). Osteoclast heterogeneity is also bone-site-specific, where osteoclasts in intramembranous bone (e.g., calvaria) respond and function differently than those in endochondral bone (e.g., long bone) (165, 167). Notably, osteoclasts in long bones exhibit differential regulation and response in trabecular and cortical bone (165). Whether osteoclast heterogeneity is due to matrix compositional variances, to diverse osteoblastic activity, to different hormonal/neuronal modulation, or to different trabecular/cortical proportion is still not clear. Osteoclast heterogeneity could be due to different precursor origin, since osteoclasts can differentiate from cells originated from bone marrow, spleen, blood stream and dendritic cells. The pre-conditioning of osteoclast precursors might also be a source of heterogeneity, as comparable number of peripheral bloods monocytes of RA patients form more osteoclasts than those of osteoporosis patients (165). Morphological heterogeneity in osteoclasts correlates to their heterogeneity in function, as smaller rounded osteoclasts with 3-4 nuclei adhere to RGD (Arg-Gly-Asp)-containing bone-residing PT (prothrombin) and TH (thrombin) while larger osteoclasts with extended cytoplasmic processes and 6-7 nuclei attach to osteopontin and fibronectin (166). These smaller osteoclasts show less resorption activity than the larger ones. Differences between osteoclasts from endochondral and intramembranous bone are listed in (Table 2) (165-167).
Table 2. Differences between endochondral and intramembranous osteoclasts. Adapted from Everts, V et al: 2009, Biochim Biophys Acta.

<table>
<thead>
<tr>
<th>Differences</th>
<th>Skull/jaw bones</th>
<th>Long bones</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPG overexpression</td>
<td>Unaffected</td>
<td>Affected</td>
</tr>
<tr>
<td>PGE2 application</td>
<td>Unaffected</td>
<td>Affected</td>
</tr>
<tr>
<td>PTH application</td>
<td>Unaffected</td>
<td>Affected</td>
</tr>
<tr>
<td>Remodeling time</td>
<td>Relatively fast</td>
<td>Relatively low</td>
</tr>
<tr>
<td>Remodeling (Pressure-induced)</td>
<td>Very sensitive</td>
<td>Less sensitive</td>
</tr>
<tr>
<td>Size</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Sympathectomy</td>
<td>Affected</td>
<td>Unaffected</td>
</tr>
<tr>
<td>TRAP expression</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>Use of CPs for resorption</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Use of MMPs for resorption</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>CD68 and CD163 expression</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>AE2 (anion exchanger 2)</td>
<td>Unaffected</td>
<td>Affected</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>Unaffected</td>
<td>Affected</td>
</tr>
<tr>
<td>Connexin 43</td>
<td></td>
<td>Unaffected</td>
</tr>
<tr>
<td>HIF-α</td>
<td>Unaffected</td>
<td>Affected</td>
</tr>
<tr>
<td>Ihh (Indian hedgehog homolog)</td>
<td>Unaffected</td>
<td>Affected</td>
</tr>
<tr>
<td>MMP-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFATc1</td>
<td>Unaffected</td>
<td>Affected</td>
</tr>
<tr>
<td>TRAF6</td>
<td>Unaffected</td>
<td>Affected</td>
</tr>
<tr>
<td>Sh3bp2 (Src homology-3 domain-binding protein 2)</td>
<td></td>
<td>Unaffected</td>
</tr>
</tbody>
</table>

Other cell types in bone tissue

B- and T-lymphocytes have a role in bone homeostasis, where they produce more than 50% of OPG in the bone marrow under physiological conditions (168).

Megakaryocytes (from hematopoietic origin) in the bone marrow have the ability to regulate both osteoblasts and osteoclasts and produce RANKL and OPG, which qualify them to have a role in maintaining bone volume (169, 170).

Osteomacs are bone tissue resident macrophages that reside on or near endosteal and periosteal surfaces. Osteomacs are suggested to play a role in regulation of osteoblast function (171).

Vascular endothelial cells are key cooperators in bone homeostasis through formation of the essential supplier capillaries. Crosstalk between vascular endothelial cells and adjacent bone cells has been suggested (172).
Regulation and crosstalk of osteoblasts and osteoclasts

Osteoblasts and osteoclasts crosstalk to ensure adequate bone homeostasis, which in turn depends on a right coupling between their functions. This communication is enabled through either direct cell-cell contact or secreted paracrine factors and small hydrophilic molecules. Communication between cells is mediated by membrane-bound ligands and receptors interaction and through gap junctions. Secreted paracrine factors, include chemokines, cytokines, growth factors (173).

Regulation of osteoclasts by osteoblasts

Osteoblasts directly regulate osteoclastogenesis by expressing M-CSF, RANKL or OPG and indirectly by secretion of either the inhibitory cytokine IL-33 or various stimulatory molecules, such as IL-1β, IL-6, PTHrP and TNF-α (76, 174). In addition, osteoblasts can regulate osteoclast formation by secreting Wnt or semaphorin proteins (76). Osteoblasts produce Sema3A (semaphorin 3A; a secreted axon guidance protein) that suppresses osteoclast differentiation by binding to its receptor Nrp1 (Neuropilin-1), which sequesters Plexin-A1 from the receptor complex Pleixn-A1/TREM-2/DAP12 on OCPs resulting in inhibited ITAM activation of NFATc1 (76). In contrast, Sema3A stimulates osteoblast differentiation via Wnt/β-catenin pathway (76). Moreover, osteoblasts express Wnt5a and Wnt16, which potentiates and inhibits RANKL-induced osteoclastogenesis, respectively, through the non-canonical Wnt signaling pathway (68, 175). Wnt5a binds to Ror2 (receptor tyrosine kinase-like orphan receptor 2)/FZD complex recruiting DSH (dishevelled) and subsequent DAMM1 (dishevelled-associated activator of morphogenesis 1), which activates c-Jun via Rac1-JNK pathway. c-Jun up-regulates RANK expression, potentiating RANKL-induced osteoclast formation (76). Wnt16 inhibits osteoclast differentiation by inhibiting NF-κB-induced NFATc1 via JNK /c-Jun cascades in a non-canonical pathway (175). Interestingly, Wnt16 has also an autocrine effect on osteoblasts that results in increased OPG expression, further inhibiting osteoclast differentiation (175). Moreover, osteoblast-secreted Wnt3a and Wnt4 negatively regulate osteoclastogenesis through the non-canonical pathways (68).
Recently, it was shown that osteoblasts communicate with osteoclasts via microvesicles containing RANKL. Microvesicles dispatched from osteoblasts merge with OCPs and stimulate osteoclast formation through specific ligands-receptor interaction (i.e. RANKL-RANK) (176).

**Regulation of osteoblasts by osteoclasts**

Osteoclasts talk back to osteoblastic lineage by either releasing matrix-embedded molecules during resorption process or secreting osteoclast-derived molecules, so called clastokines. Released matrix-embedded molecules include BMPs, TGF-β and IGF-1 and 2, which have effects on osteoblast recruitment, proliferation and differentiation (152, 174, 177). Clastokines are presented below in (Table 3)

**Table 3. Summary of clastokines and their effect on osteoblasts.**

<table>
<thead>
<tr>
<th>Clastokine</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afamin</td>
<td>promotes osteoblast migration</td>
</tr>
<tr>
<td>BMP6</td>
<td>promotes osteoblast differentiation</td>
</tr>
<tr>
<td>CTHCR1 (collagen triple helix repeat containing 1)</td>
<td>stimulates osteoblast differentiation</td>
</tr>
<tr>
<td>C3a (complement component 3a)</td>
<td>promotes osteoblast differentiation</td>
</tr>
<tr>
<td>CT-1 (cardiotrophin-1)</td>
<td>promotes osteoblast differentiation</td>
</tr>
<tr>
<td>CXCL16</td>
<td>pro-migratory factor for osteoblasts</td>
</tr>
<tr>
<td>HGF (hepatocyte growth factor)</td>
<td>Promotes osteoblast proliferation</td>
</tr>
<tr>
<td>LIF</td>
<td>osteoclast-derived LIF suppresses TGF-β1-induced migration of osteoblasts stimulates bone formation</td>
</tr>
<tr>
<td>PDGF-BB (platelet derived growth factor BB)</td>
<td>secreted by non-resorbing osteoclasts induces MSCs migration, but inhibits osteoblast differentiation</td>
</tr>
<tr>
<td>Sclerostin</td>
<td>inhibits bone formation</td>
</tr>
<tr>
<td>Sema4D (also known as CD100)</td>
<td>inhibits osteoblast differentiation</td>
</tr>
<tr>
<td>TRAP, a Purple Acid Phosphatases (PAPs) family member</td>
<td>promotes osteoblast differentiation</td>
</tr>
<tr>
<td>Wnt10b</td>
<td>promotes osteoblast differentiation</td>
</tr>
<tr>
<td>SP1 (sphingosine-1-phosphate)</td>
<td>promotes osteoblast differentiation</td>
</tr>
</tbody>
</table>
Bidirectional osteoclast-osteoblast regulation

Osteoclasts and osteoblasts not only crosstalk through paracrine signaling, but also regulate each other’s activity via cell-cell contact (174). This cell-cell signaling is facilitated through EphrinB2 on the osteoclast and its receptor EphB4 (Ephrin type-B receptor 4) on the osteoblasts (174, 178). The characteristic of Eph receptors, which are transmembrane tyrosine kinases, is the bidirectional signaling (178). Among many functions, Eph receptors have a peculiar role in bone homeostasis. Eph receptors consist of two subfamilies, EphA and EphB, which interact with Ephrins A and B superfamilies (179). Eph receptors contain an extracellular Ephrin-binding region, and an intracellular region. The intracellular part constitutes of a juxta-membrane domain, a tyrosine kinase domain, a SAM (sterile alpha motif) and a PDZ-binding domain (179). EphrinA ligands are extracellular and anchored by a glycosylphosphatidyl-inositol binding to the cell membrane, while ligands of EphrinB family are transmembrane proteins (179). The expression of EphrinB2 increases during osteoclast differentiation, but osteoblasts express both EphrinB2 and EphB4 (173, 174, 178). The ligation between EphrinB2 on the osteoclasts and its receptor EphB4 in the osteoblasts not only induces a forward signaling that promote osteoblast differentiation, but also a reverse signaling that inhibit bone resorption (173, 174, 178). In addition, EphrinB2-EphB4 signaling inhibits RANKL expression in osteoblasts in vitro, which is supported by in vivo observations of fewer osteoclasts in mice overexpressing EphB4 (174, 178). OCPs also express EphA2 and EphrinA2, which are induced by RANKL while EphA4 is only expressed on mature osteoclasts (173). Interaction of EphrinA2-EphA2 between OCPs, or between pre-osteoblasts and mature osteoclasts, enhances osteoclast formation and inhibits osteoblast differentiation (173). EphrinB2-EphA4 interaction between osteoclasts inhibits osteoclast formation (from OCPs) and osteoblast differentiation (EphrinB2-EphB2 mediated between osteoclasts and osteoblasts) (173) (Figure 18). This cell-cell regulation is likely not a major control mechanism since the mature osteoclast-osteoblast contact is infrequent and other cell types than osteoblasts exist that express EphrinB2-binding receptors, such as EphA2, EphB2 and EphB4. Moreover, the deletion of
osteoclastic EphrinB2 did not cause any phenotype \textit{in vivo} (173, 174). Ephrin signaling is also important within the osteoblastic lineage by enhancing osteoblast migration and differentiation (174). Mice lacking EphrinB1 in osteoblasts exhibit reduced bone mineral density with normal osteoclastogenesis (174). EphrinB2 in osteoblasts is possibly participating in PTH and PTHrP enhanced bone formation since its expression is strongly induced by these hormones (173, 174).

In summary, Eph/EphrinA signaling regulates osteoclast formation positively and osteoblast differentiation negatively, but in contrast, Eph/EphrinB signaling inhibits osteoclast formation and stimulates osteoblast differentiation.

\textbf{Figure 18.} Schematic illustration of Eph/Ephrin signaling. (A) Eph/Ephrin signaling between osteoclasts and osteoblasts. (B) Eph/Ephrin signaling within osteoclastic lineage. (C) Eph/Ephrin signaling within osteoblastic lineage.
Regulation of osteoblasts and osteoclasts by Hypoxia, pH and ATPs

Acidosis or pH<7 occurs when the concentration of protons (H+) rises. The body is equipped with several mechanisms to sense and balance pH alterations. In bone tissue, osteoblasts and osteoclasts express OGR1 and TDAG8, proton-sensing G-protein-coupled receptors, which responds to pH changes over the proper pH range (7.4–6.9). These receptors are involved in the regulation of Ca^{2+} fluxes in osteoblasts and osteoclasts (180, 181). Osteoblasts and osteoclasts also express ASICs 1–3 (acid-sensing ion channels 1-3) and P2X2 (receptor for ATP), while osteoblast-like cells express ion channels: TRPV4, TRPC4 and TRPM8 (transient receptor potential cation channel subfamilies V, C and M members), and osteoclasts express an inward rectifier K+ channel (180).

Acidification to pH 6.9 completely abrogates osteoblast mineralization by downregulating ALP expression and upregulating the expression of matrix Gla protein, an inhibitor of mineralization (180). pH≤6.9 does not affect osteoblast proliferation or collagen synthesis (180). Acidosis initiates (at pH<7.4) and maximizes (at pH 6.8) osteoclasts’ enduring resorption activity (180, 181). This process is associated with rapid upregulation of CA II, V-H^+-ATPase, TRAF6, cathepsin K and TRAP in osteoclasts (180). Acid-induced osteoclast activity can be further potentiated by RANKL, ATP, PTH and vitamin-D3 (180, 181).

Hypoxia or low oxygen pressure (pO_2) is a condition that occurs due to reduced or disrupted blood supply to a tissue. The body maintains the oxygen levels constant with narrow fluctuation range (180). Excessive O_2 levels may lead to generation of damaging reactive oxygen radicals while hypoxia can result in failure in energy production via ATPs (180). In normoxia (optimal O_2 levels), HIFs (transcription factor heterodimers of α and β subunits) are subjected to hydroxylation by PHD (prolyl hydroxylase) that utilizes molecular oxygen and 2-oxoglutarate in cooperation with ascorbate (180). The hydroxylated HIF (e.g., HIF-α) is polyubiquitinized under the control of pVHL (Von Hippel Lindau tumor suppressor protein; E3 ubiquitin ligase) and subsequently degraded. Hypoxia inhibits the degradation process of HIF-α, resulting in its stabilization and dimerization with the β subunit and subsequently translocation and transcriptional activity (180). Hypoxia decreases osteoblasts’ bone formation activity and completely inhibits it when pO_2 is ≤2% by arresting proliferation,
growth and differentiation of osteoblasts through suppression of Runx2 (180). Decreased collagen production is also a result of suppressed expression and activity of oxygen-dependent enzymes, prolyl hydroxylase and lysyl oxidase, essential for post-translational modification of collagen molecules (180). Moreover, hypoxia induces the expression of VEGF, IGF-2 and TGF-β1 in osteoblasts (180, 182). Interestingly, hypoxia drives the endochondral ossification (182). On the contrary, hypoxia increases osteoclast formation and activity in a PGE2 dependent manner (180). It also stimulates bone resorption via enhanced release of ATPs and GDF-15 (growth differentiation factor-15) from osteoblasts and/or osteocytes (180, 183). It is notable to mention that hypoxia gives rise to metabolic acidosis (180).

Osteoblasts and osteoclasts express both P2X and P2Y receptor subfamilies. These receptors respond to ATP, ADP and UTP (uridine triphosphate). ATPs and UTP promote osteoblast proliferation and differentiation (by enhancing Runx2), but inhibit bone mineralization (184). The inhibitory effect is mediated by intracellular signaling via P2 receptors and extracellular hydrolysis of ATP to PPi (pyrophosphate), which act as water softener and dissolves the minerals (184). Conversely, ATPs stimulate osteoclast formation, survival and bone resorptive activity (180, 184).

- Bone homeostasis

Bone remodeling
Bone is a highly specialized and dynamic tissue that undergoes modeling (reshaping) to meet and adopt to biochemical and biomechanical demands and changes. Bone is constantly renewed (remodeling) by replacing the old and microdamaged bone with new, in order to preserve its strength (185). This process is carried out in many small loci called BMU (basic multicellular unit) or BRC (bone remodeling compartment), containing osteoclasts, osteoblasts, osteocytes, reversal cells and bone lining cells (185). A network of capillaries supplies the BRC with blood containing oxygen and nutrients, and is involved in
removing resorption products. Bone remodeling is an extremely complex, organized, controlled and coordinated process, which can be distinguished in five sequential phases that can last for six months (185) (Figure 19).

Activation Phase: The first stage is the activation of the bone surface by an initiating signal that induces the bone lining cells to digest the endosteal membrane/non-mineralized collagen by collagenases (e.g., MMP) exposing the RGD-binding sites in bone extracellular matrix (186, 187). Bone lining cells then retreat and give entrance to osteoclasts (148, 186). This stage includes recruitment of OCPs from the circulation and activation of osteoclastogenesis by lining cells/osteoblasts and osteocytes. The recruitment of OCPs is facilitated by various chemoattractants like collagen type I, osteocalcin and osteoblastic MCP-1, MIP-1α, RANTES, and MCP-3 (148, 186-188). OCPs attach to bone lining cells via ICAM-1/VLA (intercellular adhesion molecule-1; also known as CD54)/ (very late antigen; an integrin α-subunit) interaction (189). The initiating signal can be mechanical, hormonal or inflammatory (via cytokines) stimuli (186-188).

Figure 19. Schematic illustration of bone remodeling in the BRC (190).
Resorption phase: mature osteoclasts anchors to RGD-binding sites via integrins (αvβ3), reorganize their cytoskeleton and create the sealing zone, which isolates the resorption microenvironment beneath the osteoclasts (102, 186, 187). Osteoclasts pump hydrogen ions (protons) into the resorption zone, dissolving the mineral crystals from bone matrix and afterwards secret a collection of proteolytic enzymes, including MMPs, TRAP and cathepsin K to degrade the remaining organic bone matrix (102, 186, 187). Osteoclasts take up (by endocytosis) the waste products and release them via its functional secretory domain, which allows the release of the imbedded growth factors from the matrix (102, 186, 187). The resorption lacunae created by osteoclasts is an irregular scalloped cavity called Howship’s lacunae. This phase takes approximately 2 weeks during each remodeling cycle (186, 187).

Reversal phase is initiated when the retreated bone lining cells differentiate to reversal cells and colonize the resorption track forming the canopy of BRC. Reversal cells also clean the remaining undigested eroded bone matrix and form osteopontin-rich cement lines along the resorption track, which facilitates osteoblast attachment (99, 100, 186, 187). Canopy formation is essential for subsequent bone formation. Loss of canopy leads to imbalances in coupling process, which results in clearly reduced bone formation despite the increased resorption, e.g., in Cushing disease, multiple myeloma and postmenopausal osteoporosis (186, 191). Reversal/bone lining cells have the ability to proliferate (101). Recruited pre-osteoblasts and osteoblasts, by mean of coupling factors, start the bone formation process. This phase takes approximately 4–5 weeks during each remodeling cycle (186, 187).

Formation and mineralization phase begins, upon signals from coupling factors, with osteoblast recruitment and differentiation. Then, mature osteoblasts attach to the clean eroded bone surface matrix and start the deposition of osteoid (186, 187). Osteoblast recruitment into the BRC have three possible routes: either from reversal cells that transdifferentiate into bone forming osteoblasts, or by migration and transdifferentiating of canopy cells into mature osteoblasts, osteoblast progenitors from bone marrow via capillaries, or a combination of these routes (101). The deposited osteoid comprises of collagen type I, lipids and non-collagenous proteins like proteoglycans, glycosylated
proteins (e.g., ALP, SIBLING proteins (small integrin-binding ligand)) and Gla-containing proteins (e.g., matrix Gla-protein and osteocalcin). During the formation of bone lamella, some osteoblasts remain in the deposited matrix and become imbedded osteocytes interconnected by canaliculi (186, 187). Late during this phase, which takes approximately 3–4 months during each remodeling cycle; osteoblasts start to mineralize the matrix (186, 187).

Termination phase, when equal amount of resorbed bone is replaced, the remodeling cycle ends and remaining osteoblasts either undergo apoptosis or become lining cells. The mechanism underlying termination process is unknown. Bone remodeling follows the circadian rhythm with greater resorption rate at night. Fasting diminishes the bone remodeling rhythm (186, 187).

When the balance between bone resorption and formations is disrupted (pathological) due to disturbed coupling, or excessive/continuous stimuli or lack of inhibition, the net result is either a porotic or sclerotic bone.

**Bone modeling**

Physiological or pathological uncoupled bone formation and resorption is called bone modeling and occurs in situations like growth, loading, unloading/immobilization and/or inflammation. GH (Growth hormone), IGF-1 and sex hormones control bone modeling. Pathological bone modeling can occur in conditions like osteopetrosis, renal osteodystrophy and acromegaly. Bone modeling is also a predetermined process (187).

- **Regulation of bone homeostasis**

**Bone and central nervous system**

Brain is the central core regulating and controlling all organs in the body. The brain controls bone homeostasis in a complex way. Brain produces many neurotransmitters and hormones directly or via other brain-regulated organs. It regulates bone mass by three mechanisms: directly through hypothalamic peptide secretion that act on bone (192), indirectly via neuroendocrine-regulated
pituitary gland by hypothalamus (193) or by SNS (sympathetic nervous system) mediated neuronal signals (194). The brain can also mediate the upstream signals generated in the peripheral tissues like leptin from the adipose tissue (193).

Leptin (an adipocyte-derived hormone) regulates bone mass via serotonin in VMH (ventromedial hypothalamus) through norepinephrine-derived signals in SNS on osteoblastic Adrβ2 (adrenergic receptor β2), which increases RANKL expression through ATF4/CREB signaling pathway and subsequent bone resorption (192, 193).

Hypothalamus controls bone mass by regulating pituitary gland to secrete GnRH (gonadotropin-releasing hormone) that act on gonads (testis and ovaries), resulting in secretion of estrogens, androgens and progesterone via FSH (gonadotropins follicle-stimulating hormone) and LH (luteinizing hormone) (195). In turn, sex hormones directly regulate bone mass. Under the surveillance of hypothalamus, pituitary gland releases oxytocin that stimulates osteoblastogenesis and bone formation by enhancing BMP-2, ATF4 and OSX expression (195). Oxytocin has a dual effect on osteoclasts, but its net effect is osteogenic (195). Prolactin affects osteoblast proliferation and Ca^{2+} turnover. Prolactin exerts its effects on the skeleton in an age-dependent manner (195). In addition, pituitary gland secretes GH that directly stimulates bone and indirectly via IGF-1 from the liver (192, 195). Moreover, under the regulation of hypothalamic TRH (thyrotropin releasing hormone), pituitary gland secretes TSH (thyroid stimulating hormone), which stimulates secretion of the thyroid hormones T3 and T4 that contribute in regulating bone mass (192). Notably, TSH itself has a direct effect on bone (192).

Hypothalamus secretes neuropeptides into the blood stream that directly act on bone, including α-MSH (α-melanocyte-stimulating hormone, also known as α-melanotropin), CART (cocaine-and amphetamine-regulated transcript), cannabinoids, neuromedin U and neuropeptide Y (196) (Figure 20).
Figure 20. Schematic illustration of central nervous system’s control of bone mass (192).

**Bone and peripheral sympathetic nervous system**

Bone innervation follows Hilton’s rule implying that muscle and skin innervation extends and continues to the underlying long bones and joints. Large nerve bundles alongside with the major arteries entre the long bone at multiple locations (194). Bone marrow space is innervated primarily by sympathetic vasomotor while cortical and periosteal bones are richly innervated by dense networks of sensory nerves, sensitive to both mechanical stimulation and pain, but sympathetic fibers are also present (194). Whether nerves in the periosteum fully penetrate to the marrow space is unknown, but many of these nerves penetrate into the cortical bone together with the Sharpey’s fibers, which indicates sensory perception within the cortical bone (194). The nerve distribution pattern within the bone microenvironment suggests that nerve terminals are in direct contact with a limited number of bone cells. Therefore, further signal transduction is most likely enabled by communication via intercellular GAP junctions (194). Sema3A is believed to regulate the innervation of bone, which could represent a mechanism for how developing bones control their own innervation (194). Release of epinephrine (adrenalin) and norepinephrine (noradrenalin) from sympathetic nerves activates postsynaptic ARβ2 on osteoblasts, osteocytes and chondrocytes (193, 194). Sympathetic signaling via ARβ2 in bone homeostasis might be a co-factor in increased bone
resorption induced by increased endogenous or high-dose exogenous glucocorticoids, since glucocorticoids enhance the expression of ARβ2 in osteoblasts (193, 194). Sympathetic signaling via ARβ2 might also be an interesting co-factor in osteoporosis since osteoporosis and severe depression are associated and sympathetic outflow increases due to depression (194).

Moreover, cannabinoids (e.g., anandamide and 2-arachidonolglycerol) released by sympathetic nerves also regulate bone homeostasis by binding to CB1, CB2 and CPR55 (G-protein-coupled receptors) on the cell membranes of osteoblasts and osteoclasts, through activation of cAMP, NF-κB, and MAPK. Osteoporosis is also associated with polymorphism in the gene encoding for CB2 receptor (194, 196).

Interestingly, inhibition of sympathetic signals (sympathectomy) affects bone resorption in intramembranous bone, but not in long bone (197). In addition, bone marrow, close to trabecula, is subjected to parasympathetic innervation from PSNS (parasympathetic nervous system) that contribute to bone modeling during growth under central CNS control by inhibiting sympathetic outflow in bone (193, 194).

**Bone and hormones**

Bone cells express receptor for hormones, which affect bone homeostasis by either regulating bone formation or bone resorption.

*Sex hormones* (androgens and estrogens) play an essential role in bone growth spurts during puberty and in the maintenance of bone homeostasis. Androgens signal via AR (androgen receptor) and estrogens through ERs (estrogen receptors α and β) in two ways, the genomic and non-genomic pathways (198, 199). Sex hormones diffuse into the nucleus where they activate the receptors by hormone-independent phosphorylation and the hormone-receptor complex binds directly or in cooperation with other co-regulatory factors to ARE (androgen response element) or ERE (estrogen response element) and start the transcription of targeted genes (198, 199). Calveoli (a mechanotransduction site) can transport sex-hormone receptors to the cell membrane, where in the absence of the hormones, growth factors like EGF (epidermal growth factor) and IGF-1, cAMP and PKA phosphorylate them (200). In this non-genomic pathway, sex
hormone-receptors can upon activation by sex hormone, interact with Src and IGF-1R (IGF-1 receptor) to amplify the downstream signaling through ERK1/2 (198-200) (Figure 21). Androgens stimulate osteoblastogenesis and bone formation potently while estrogens decrease osteoclast formation and activity and increase their apoptosis (198, 199). Estrogens’ positive effects on osteoblasts are dependent on the stage of osteoblast differentiation (198, 199). The androgen receptors on osteoblasts and osteocytes are essential for trabecular bone maintenance, but favor periosteal bone formation in males (198, 199). Loss of estrogen in postmenopausal women affects the osteoblastic cells and monocytes, resulting in elevated expression of cytokines like RANKL, IL-1, IL-6, M-CSF and TNF-α and subsequent enhanced osteoclast formation and activity and thereby increased bone resorption (198, 199). In addition, estrogen depletion decreases osteoblasts’ survival and activity (198, 199).

Figure 21. Schematic illustration of sex-hormone’s genomic and non-genomic signaling pathways (200). SR: Sex hormone receptor; SRE: SR-response element; TF: transcription factor; CR: co-regulators; TRE: TF-response element; TMR: transmembrane receptor; P: phosphorylation.
Parathyroid hormone (PTH) is a peptide produced in the parathyroid glands. Its role is to maintain the serum calcium homeostasis, which is achieved by affecting directly bone and kidney, and indirectly the intestine (201). Calcium-sensing receptor (CaSR) in the parathyroid cell membrane regulates PTH synthesis and release (201). Low Ca\(^{2+}\) concentration triggers PTH synthesis and secretion while high concentration of Ca\(^{2+}\) inhibits PTH release and synthesis (201). To a lesser extent, secretion of PTH is stimulated by increased phosphate concentration in the serum (201). PTH exerts its effect directly on osteoblasts and osteocytes to either stimulate bone formation or osteoclast formation and activity via increased cytokine secretion (202, 203). The outcome of PTH action on bone, increased formation or resorption, is dependent on its dose and periodicity. Continuous PTH secretion promotes bone resorption while intermittent and low dose of PTH favors bone formation (202, 203). T cells appear to have a permissive role in both PTH-induced bone resorption and bone formation (202, 203). PTH promotes bone resorption by enhancing RANKL/OPG ratio and stimulates bone formation by enhancing EphrinB2/EphB4 signaling in osteoblasts and by inhibiting Dkk1 and sclerostin expression (two inhibitors of Wnt signaling) (202, 203). PTH also increases proliferation and differentiation of osteoblasts, while decreasing their apoptosis. In addition, PTH controls phosphate metabolism through inhibiting reabsorption of phosphate in the kidneys (202, 203). It is suggested that PTH regulates the formation of 1,25(OH)\(_2\) vitamin D\(_3\) in the kidney, which then stimulates calcium absorption in the intestine (204).

PTHrP (parathyroid hormone-related protein), produced by malignant tumor cells, exhibit similar properties as PTH and is the main humoral mediator of malignancy-associated hypercalcemia and local stimulator of osteoclast formation in certain osteolytic bone metastases of malignant tumors (205, 206).

Calcitonin (CT) is a potent osteoclast-inhibitory peptide, produced in and secreted from parafollicular C cells of thyroid gland. Its secretion is regulated by serum Ca\(^{2+}\) concentration. Conversely, compared to PTH, hypercalcemia enhances the secretion of CT, which inhibits bone resorption and promotes excretion of calcium and phosphate to restore their homeostasis (207).
Other hormones like, growth hormone, glucocorticoids, thyroid hormone (T3), thyroid-stimulating hormone (TSH) and insulin also affect bone modeling. Interestingly, T3 modulates bone mass through interaction with sympathetic nervous system (208).

**Bone and vitamins**

*Vitamin A* is important for bone health, but its role is controversial. Vitamin A is provided by dietary intake as either pre-formed vitamin A (retinol or retinylesters) from animal source or as pro-vitamin A (carotenoids) from vegetarian source (209). Biologically active vitamin A is the converted compound called ATRA (all-trans-retinoic acid), which binds to intracellular nuclear receptors, including RARα, RARβ or RARγ (retinoic acid receptors), which heterodimerize with RXRα, RXRβ, RXRγ (retinoid X receptors) for which the ligand is unknown (orphan receptors) (210). The formed complexes function as ligand-activated DNA-binding transcription factors (210). Elevated levels of vitamin A (retinol or retinylesters) in the serum is associated with bone fragility and fracture risk, while carotenoids are associated with improved bone strength (209). In addition, ATRA exhibits a bimodal effect on osteoclastogenesis, where it enhances periosteal osteoclast formation and bone resorption, but inhibits osteoclast formation in bone marrow (210). Contradicting reports exist regarding the effects of hypervitaminosis A (abnormally high level of the vitamin in the serum) on bone formation (209). Carotenoids are suggested to exert their beneficial effect on bone as antioxidants (209).

*Vitamin D* is undoubtedly crucial for bone health. Vitamin D deficiency severely affects bone mineralization and causes osteomalacia (Rickets, during growth) and low bone mineral density (BMD; osteoporosis, in the elderly) (211). Vitamin D is produced from cholesterol in the skin upon exposure to sun light and is activated in the kidney to 1,25(OH)2-vitamin D3 (212). Vitamin D can be supplemented by dietary intake. It primarily regulates intestinal calcium uptake, participating in calcium and phosphate homeostasis and thus, prevent bone resorption (211, 212). Furthermore, 1,25(OH)2-vitamin D3 induces bone mineralization and formation when accompanied by an adequate intake of dietary calcium (211, 212). Conversely, hypervitaminosis D accompanied by
insufficient dietary calcium intake results in decreased bone mineralization and enhanced bone resorption in order to maintain calcium homeostasis in the serum (211, 212). 1,25(OH)2-vitamin D3 binds and activates its intracellular receptor VDR (vitamin D receptor) forming a DNA-binding nuclear transcription factor complex together with RXRs and other co-activators (211, 212). Activation of VDR stimulates RANKL/OPG ratio in osteoblasts and thereby bone resorption. It also has roles on osteoblast proliferation, differentiation and mineralization (213), as well as on osteocytic production of FGF23 (214). Furthermore, it is suggested that 1,25(OH)2-vitamin D3 has an inhibitory effect on osteoclast differentiation and formation, but not on the resorptive ability of mature osteoclasts (215). However, vitamin D affects bone differentially dependent on its own status as well as mineral levels in a coordinated network of bone, kidney and intestine. (211, 212).

Vitamin E also plays a role in bone homeostasis by promoting osteoclast fusion through enhanced DC-STAMP expression in a p38- and MITF dependent way and thereby promotes bone resorption independent of its antioxidant feature (216). In contrast, vitamin E has beneficial effects in osteoporosis, including decreased osteoclast number and enhanced osteoblast number and function and consequently improved BMD (217, 218). The positive effects is likely due to the antioxidant and anti-inflammatory capacities of vitamin E by suppressing mevalonate/GTPase pathway and inducing bone formation-related genes (218). The effect of vitamin E appears to be bimodal and concentration-dependent, which also interacts with other vitamins (218).

Vitamin K is accessory in bone homeostasis as well. It is a potential protector against age-related bone loss by acting as a co-factor for gamma-carboxylation of osteocalcin (called bone Gla-protein; essential for Ca-binding) or the SXR-RXR complex activation (steroid and xenobiotic receptor) and subsequent enhanced collagen accumulation (219). Moreover, vitamin K has an inhibitory effect on renal calcium excretion as well as on osteotropic PGE2 and IL-6 expression (219). Vitamin K is provided by dietary intake and has two isoforms, vitamin K1 and K2, and believed to have beneficiary effects on BMD and fracture healing. It is suggested that vitamins K and vitamin D synergistically modulate bone homeostasis (220).
Vitamin B complex levels are also suggested to be associated with osteoporosis, while vitamin C is essential for osteoblast activity and thus normal bone development. In addition, Vitamin C is correlated with enhanced BMD in osteoporotic postmenopausal women (220).

**Bone and kidney**

Kidney is a very important organ participating in network of bone homeostasis by regulating calcium and phosphate concentration in the serum (221). In addition, kidneys activate vitamin D and influence bone tissue while responding to osteocyte-produced FGF23 (222) and sclerostin (223). Renal dysfunction may cause secondary hyperparathyroidism, which also affect bone negatively by enhancing cortical bone resorption (221, 224).

**Bone and adipose tissue**

Adipose tissue and bone are interrelated, where adipocytes and osteoblasts have the same origin and a reciprocal relationship; i.e. osteoblast differentiation has an inverse effect on adipocytes’ development and vice versa (225). Moreover, differentiation of MSCs in the bone marrow towards adipogenesis is correlated with osteoporosis (225). In addition to inflammatory molecules secreted by adipose tissue, such as CRP, IL-6, TNF-α, adipokines (adipocyte-derived molecules), including adiponectin, leptin, resistin, and visfatin, play a role in bone and cartilage homeostasis (225, 226). Among adipokines, adiponectin has a direct effect on osteoblasts’ activity and thus alters bone homeostasis (227). Using adiponectin knock out mice, it has been found that adiponectin decreases bone mass and osteoclast formation (228), most likely indirectly through an effect on osteoblasts, increasing RANKL/OPG ratio (229). In humans, high serum adiponectin predicts incident fractures (230). However, conflicting reports exist showing an osteogenic role for adiponectin (231). Overexpression of adipogenic transcription factor Zfp467 increases RANKL expression in stromal cells supporting osteoclastogenesis both *in vitro* and *in vivo* (232). Moreover, active Brown adipose tissue (BAT) is positively associated with BMD (225).
Bone and gut microbiota

Autoimmune and inflammatory disorders related to the gut have been connected with low bone mass, indicating a link between the gut and bone (233). Gut microbiota, the symbiotic commensal bacteria (and fungi) of the gastrointestinal tract are known for their beneficial role in the host’s health, like protection against invading pathogens, facilitating food digestion and absorption, and shaping the immune system(233). Gut microbiota and its composition are determinant of bone mass (233). Since it is impossible to exist as germfree, the composition of gut microbiota is important in bone homeostasis, especially in postmenopausal women (233). It stimulates bone resorption by affecting and preparing the immune system, which regulates osteoclast formation. In the light of this knowledge, probiotics (beneficial commensal live bacteria or fungi) have been suggested as a treatment for postmenopausal osteoporosis (233).

Bone and mechanical stimuli (loading or unloading)

Mechanical strain (loading) stimulates and maintains bone mass, while unloading (immobilization) favors bone loss. Interestingly, bone responds to dynamic, but not static strain. Osteocytes as mechano-sensors in bone tissue are responsible for regulation of bone homeostasis. Osteocytes utilize several signaling pathways to orchestrate the mechanical stimuli-induced bone regulation, including IGF-1, nitric oxide, prostaglandin and Wnt/β-catenin pathway. In addition, sex hormones contribute to loading-induced osteogenesis. Interestingly, ARs (Androgen receptors) and ERα, but not ERβ (Estrogen receptors) in non-osteocytic cells mediate this effect in males and females (mice), respectively. However, ERα in early osteoblasts is crucial for mechanical strain-induced bone formation in females (200, 234). The role of ERα in the loading response of cortical bone is observed also in absence of estrogen, most likely due to ligand independent phosphorylation of the receptor (235). Interestingly, the expression of OSM, a potent and important stimulator of osteoblast differentiation and bone formation, is significantly upregulated in both osteoblastic cells, macrophages and T cells, indicating a possible endocrine mechanism in loading-induced osteogenesis (236).
**Bone and immune system (Osteoimmunology)**

Osteoimmunology, a term referring to the connection and interplay between bone and immune system, was coined in year 2000 by Dr. Aaron and Dr. Choi, but was first discovered in year 1972 by Dr. Horton and colleagues when they observed osteoclast formation and activity triggered by supernatant fluid from human leukocytes cultures (237, 238).

Osteoclasts and immune cells share the same origin in myeloid lineage and in fact, osteoclasts are specialized giant macrophages and the stimulatory cytokine for macrophage differentiation and survival, M-CSF, is also necessary for osteoclast differentiation (239). Immune cells express inflammatory cytokines that promote osteoclastogenesis and the key factor in osteoclastogenesis, RANKL, was first identified as dendritic cell regulator secreted by T cells (239). Excessive and aberrant activity of immune cells in autoimmune conditions, like RA results in bone destruction due to enhanced osteoclast formation and function. B cells, T cells and NK (natural killer) cells express RANKL and T helper subset 17 (T_{H17}) cells in RA express IL-17, which activates synovial fibroblasts to express RANKL promoting bone lysis (239). In contrast, immune cells, including T cells express IL-4, IL-10, IFN-γ and OPG inhibiting osteoclastogenesis. These observations indicate a profound interaction between immune system and bone (239) (**Figure 22**).

**Figure 22.** Schematic illustration of interaction between osteoclasts and immune cells (239).
**Shared mechanisms by immune system and bone**

Deficiency in the osteoclastogenic indispensable RANK-RANKL mechanism causes defect secondary lymphoid tissue (peripheral lymph nodes) development and organization (240). RANKL is involved in pathogenicity of inflammatory bowel disease through regulation of dendritic cells and in induction of pancreatic regulatory T cells (Tregs) in diabetes (240). In addition, RANK-RANKL mechanism is suggested to mediate UV light-induced immune suppression by inducing Tregs production via keratinocyte-Langerhans cell axis (240). Moreover, RANK-RANKL in association with CD40 establishes self-tolerance by promoting the development of autoimmune regulator (AIRE)-expressing mTECs (medullary thymic epithelial cells) as AIRE-reactive T cells are eliminated from thymus (130, 240). Furthermore, RANKL is essential in B cell development and T cell activation (130).

Dendritic cells and T cells share signaling adapter TRAF6 and transcription factor NFATc1, respectively, with osteoclasts. In addition, cathepsin K inhibition suppresses both autoimmune inflammation (i.e. dendritic cells mediated) and osteoclast mediated bone destruction (239). Osteoclasts and immune cells, including dendritic cells, macrophages, neutrophils, NK cells, B and T cells commonly utilize ITAM in their intracellular signaling (130, 240).

**Regulation of osteoclastogenesis by immune cells**

Although, T cells express RANKL, activated T cells suppress osteoclastogenesis by expressing various cytokines such as IL-4, IL-10 and IFN-\(\gamma\) (130) and cell-cell contact by CTLA-4 (cytotoxic T-lymphocyte antigen-4) binding to B7-1 and B7-2 (174). Th1, Th2 and Treg cells exert inhibitory effects on osteoclast formation, while B and Th17 cells promote osteoclastogenesis (130). In addition, TLRs activation in immune cells, among others, regulates osteoclast formation and activity (241). Interestingly, macrophage depletion leads to endosteal osteoblast loss (242).
**Regulation of immune system by bone tissue**

Bone harbours hematopoietic stem cells niche in the marrow and contributes to its regulation. Molecules generated by bone cells affect immune system, like cathepsin K, CXCL12, GM-CSF, matrix glycoproteins, osteopontin and TRAP (130, 242). TRAP deficiency causes autoimmunity by accumulating phosphorylated osteopontin and subsequent activating TLR signaling in dendritic cells (130). Cathepsin K also enhances TLR-activation in DCs, resulting in enhanced IL-6 and IL-23 expression (130). Osteoclasts are suggested to act as antigen presenting cell and activate CD4+ and CD8+ T cells (243). Osteoblasts can regulate activation of immune by acting as antigen presenting cells (244). Osteocytic sclerostin impairment results in reduced B cells number due to increased apoptosis (245). In addition, osteoblasts modulate HSC niche by expression of jagged-1 and PGE2 (242). Furthermore, HSC niche is regulated by CXCL12, angiopoietin-1, SCF (also known as kit ligand) and TGF-β (242). Osteoclasts are also suggested to contribute in regulation of HSC niche (246, 247). Furthermore, immune system mediates the effect of gut microbiota on the skeleton.

In summary, bone regulation involves a multilayered and complex network with immune system.
- **Bone, infection and inflammation**

In response to the presence of pathogens or foreign materials, the body mobilizes the immune system by secretion of various signaling molecules in order to clear the infection. In autoimmune conditions, these molecules are released in misrecognition of self-structures. These secretory signaling substances include chemokines, cytokines, interferons, prostaglandins and other signaling molecules (e.g., DKK1) (248). Malignant conditions can also affect bone tissue by secreting hormones, growth factors and other signaling molecules (249). The clinical manifestation of these conditions on bone is more often osteolytic, but sometimes sclerotic (248, 249). This is due to perturbed balance between local bone resorption and bone formation, which in turn depends on the balance between released stimulatory and inhibitory factors affecting the bone cells (248, 249). Rheumatoid arthritis, psoriasis arthritis, loosened joint prosthesis, periodontitis and peri-implantitis are conditions characterized by inflammation-induced bone loss (248), while breast cancer and multiple myeloma are examples of malignancy with osteolytic lesion (249). Cytokines/chemokines, prostaglandins, hormones or other signaling molecules released by resident, immune or cancer cells affect bone tissue differently and therefore the outcome of inflammatory mediators on bone is likely dependent on the complex balance between the inhibitory and stimulatory signals in bone cells (248, 249). In inflammatory conditions, secreted or expressed inflammatory factors usually cause osteolysis by stimulating osteoclasts and inhibiting osteoblasts, but increased osteoblastogenesis and subsequent bone formation has also been observed (248, 250). Moreover, malignant cells release factors that stimulate osteoclasts formation and activity and inhibit osteoblastogenesis, but in some cases, they favor bone formation (249, 251). A summary of inflammatory molecules and their mode of action on bone tissue and cells is presented in (Table 4) and (236, 252-254) (Figure 23).
Table 4. A summary of cytokines and other molecules their effect on bone metabolism.

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Expressed by</th>
<th>Effect on bone</th>
<th>Effect on osteoblasts</th>
<th>Effect on osteoclasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α and IL-1β</td>
<td>Immune cells, osteoblastic cells, fibroblasts, endothelial cells, epithelial cells</td>
<td>Important for osteogenesis during growth. Stimulates bone resorption in inflammation</td>
<td>Enhance RANKL-release in a STAT3-dependent way</td>
<td>Prolong mature osteoclast survival, induce osteoclastogenesis in committed OCPs</td>
</tr>
<tr>
<td>IL-4 and IL-13</td>
<td>T&lt;sub&gt;H&lt;/sub&gt;2 cells, basophils</td>
<td>Inhibit bone resorption</td>
<td>Inhibit RANKL and inflammatory cytokine release in a STAT6 dependent manner, enhance OPG expression</td>
<td>Inhibit osteoclast formation and activity</td>
</tr>
<tr>
<td>IL-6</td>
<td>Immune cells, adipocytes, neural cells, osteoblastic cells, fibroblasts, endothelial cells, epithelial cells</td>
<td>Reduces osteoblast number. Stimulates bone resorption in inflammation</td>
<td>Enhances RANKL-release in a STAT3-dependent way</td>
<td>Induces osteoclast formation and activation</td>
</tr>
<tr>
<td>IL-7</td>
<td>osteoblastic cells, fibroblasts, endothelial cells, epithelial cells</td>
<td>Enhances physiological trabecular bone formation in females. Stimulates bone resorption in inflammation</td>
<td></td>
<td>Negative regulator in trabecular bone, but stimulates osteoclast formation through activation of T cells</td>
</tr>
<tr>
<td>IL-8</td>
<td>Tumor cells</td>
<td>Stimulates bone resorption in inflammation</td>
<td>Enhances RANKL-expression</td>
<td>Induces osteoclastogenesis, possibly independent of RANKL</td>
</tr>
<tr>
<td>IL-10</td>
<td>Immune cells</td>
<td>Inhibits bone resorption</td>
<td></td>
<td>Inhibits osteoclast formation, but no effect on osteoclast activity</td>
</tr>
<tr>
<td>IL-11</td>
<td>Osteoblasts</td>
<td>Stimulates bone resorption in inflammation</td>
<td>Enhances RANKL-expression</td>
<td>No direct effect, but important for normal osteoclast formation</td>
</tr>
<tr>
<td>IL-12</td>
<td>Immune cells</td>
<td>Inhibits bone resorption</td>
<td></td>
<td>Inhibits osteoclast formation by inducing apoptosis</td>
</tr>
<tr>
<td>IL-15</td>
<td>Immune cells, osteoblastic cells, fibroblasts, epithelial cells, neural cells</td>
<td>Stimulates bone resorption in inflammation</td>
<td></td>
<td>Co-stimulator of osteoclastogenesis, but important for physiological osteoclast formation</td>
</tr>
<tr>
<td>Molecules</td>
<td>Expressed by</td>
<td>Effect on bone</td>
<td>Effect on osteoblasts</td>
<td>Effect on osteoclasts</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
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<td>--------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>IL-17</td>
<td>T(_{h}17) cells</td>
<td>Stimulates bone resorption in inflammation</td>
<td>Enhances RANKL-production via PGE synthesis in Act1-dependent way</td>
<td>Indirectly stimulates osteoclastogenesis by enhancing RANKL or osteotropic cytokines</td>
</tr>
<tr>
<td>IL-18</td>
<td>Immune cells, osteoblastic cells, fibroblasts</td>
<td>Stimulates bone resorption in inflammation <em>in vitro</em></td>
<td><em>in vitro</em> Enhances GM-CSF and IFN-γ</td>
<td><em>in vitro</em> inhibits osteoclast formation and activity <em>in vivo</em> enhances osteoclastogenesis</td>
</tr>
<tr>
<td>IL-20</td>
<td>Monocytes, epithelial cells</td>
<td>Stimulates bone resorption in inflammation</td>
<td>Enhances RANKL-expression</td>
<td>Important for osteoclast formation</td>
</tr>
<tr>
<td>IL-21</td>
<td>Immune cells, fibroblasts</td>
<td>Stimulates bone resorption in inflammation</td>
<td>Possibly enhances RANKL-expression</td>
<td>Induces osteoclast formation directly and indirectly</td>
</tr>
<tr>
<td>IL-22</td>
<td>Immune cells, fibroblasts</td>
<td>Possibly enhances RANKL-expression</td>
<td>Stimulates osteoclastogenesis indirectly</td>
<td></td>
</tr>
<tr>
<td>IL-23</td>
<td>Immune cells, fibroblasts</td>
<td>Stimulates bone resorption in inflammation</td>
<td>Possibly enhances RANKL-production</td>
<td>Indirectly induces and directly potentiates osteoclast formation</td>
</tr>
<tr>
<td>IL-27</td>
<td>Immune cells</td>
<td>Inhibits bone resorption</td>
<td>No effect</td>
<td>Inhibits/reduces osteoclast formation by IFN-γ through a STAT1 and STAT3-dependent way</td>
</tr>
<tr>
<td>IL-32</td>
<td>Immune cells</td>
<td>Unclear, stimulates cartilage destruction</td>
<td>Stimulates non-functional osteoclast formation, potentiates RANKL induced osteoclastogenesis</td>
<td></td>
</tr>
<tr>
<td>IL-33</td>
<td>Immune cells, osteoblastic cells, fibroblasts</td>
<td>Regulatory effect on physiological remodeling in trabecular bone</td>
<td>Inhibits/reduces osteoclast formation</td>
<td></td>
</tr>
<tr>
<td>IL—34</td>
<td>Large variety of cells</td>
<td>Promotes bone resorption</td>
<td>Enhances OCPs proliferation and survival like M-CSF</td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td>Large variety of cells</td>
<td>Promotes bone resorption</td>
<td>Enhances OCPs proliferation and survival</td>
<td></td>
</tr>
<tr>
<td>LIF</td>
<td>Osteoblastic cells, fibroblasts</td>
<td>Stimulates bone resorption in inflammation</td>
<td>enhances RANKL-production</td>
<td>Stimulates osteoclast formation</td>
</tr>
<tr>
<td>Molecules</td>
<td>Expressed by</td>
<td>Effect on bone</td>
<td>Effect on osteoblasts</td>
<td>Effect on osteoclasts</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------------------------------------</td>
<td>------------------------------</td>
<td>-------------------------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>OSM (catabolic effect)</td>
<td>Osteoblastic cells, fibroblasts</td>
<td>Stimulates bone resorption in inflammation</td>
<td>enhances RANKL-production in a STAT3-dependent way</td>
<td>Stimulates osteoclast formation</td>
</tr>
<tr>
<td>OSM (anabolic effect)</td>
<td>Immune cells osteoblastic cells, fibroblasts, tumor cells</td>
<td>Stimulates</td>
<td>Suppresses osteocytic sclerostin, enhances osteoblastogenesis in stromal cells</td>
<td></td>
</tr>
<tr>
<td>CT-1</td>
<td>Osteoblastic cells, fibroblasts</td>
<td>Regulates both bone resorption and formation</td>
<td>Enhances RANKL-production.</td>
<td>Stimulates osteoclast formation</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Large variety of cells</td>
<td>Stimulates bone resorption in inflammation</td>
<td>Enhances RANKL-production.</td>
<td>Stimulates osteoclast formation</td>
</tr>
<tr>
<td>IFNs</td>
<td>Large variety of cells</td>
<td>Inhibit and stimulate bone resorption</td>
<td>Inhibit and stimulate osteoclastogenesis</td>
<td></td>
</tr>
<tr>
<td>TGF-α</td>
<td></td>
<td>Stimulates bone resorption in inflammation</td>
<td>stimulate osteoclastogenesis</td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td></td>
<td>Stimulates</td>
<td>stimulate osteoclastogenesis</td>
<td></td>
</tr>
<tr>
<td>PGE</td>
<td>Large variety of cells</td>
<td>Inhibit and stimulate bone resorption</td>
<td>Inhibit and stimulate osteoblastogenesis</td>
<td>Inhibit and stimulate osteoclastogenesis</td>
</tr>
</tbody>
</table>

**Figure 23.** Schematic illustration of cytokines effects on bone cells (252).
**Toll-like receptors (TLRs)**

In 1985, Dr. Nüsslein-Volhard discovered a gene responsible for embryonic dorsal-ventral polarity (back-front orientation) of the fruit fly (*Drosophila melanogaster*). In the moment of the discovery, she spontaneously shouted out “Das ist ja Toll” in German, which means, “this is amazing/awesome”. That is where the gene got its name. Later, she and her colleague, Dr. Eric Wieschaus received Nobel Prize for this discovery (255).

In 1996, Dr. Hoffmann and colleagues found that Toll receptor had also an essential role in the susceptibility to, and combating fungal infection in fruit flies, which he was rewarded for with Noble Prize (256).

The mammalian homologues of *Drosophila* Toll called Toll-like receptors (TLRs) are single trans-membrane and non-catalytic receptors that are highly-conserved during evolution and expressed in bacteria, fungi, plants, insects and animals (including humans). Dr. Medzhitov and colleagues identified the first mammalian homolog in 1997 as hToll (now known as TLR4). The function of TLRs (*i.e.* TLR4) was discovered by Dr. Beutler in 1998, which he was honored for with Noble Prize (256).

**Morphology, position and function of TLRs**

Variety of cell types expresses TLRs. Todays, 10 human TLRs (TLR1-TLR10) and 12 mouse TLRs (TLR1-TLR9, TLR11-TLR13) have been identified (257). The expression of TLRs is not equivalent in all species, where mice lack TLR10, but express additional TLR11, TLR12 and TLR13, *Takifugu* pufferfish express TLR14 and chicken express TLR15 and TLR21 (258, 259). Interestingly, chicken expresses also isoforms and isotypes of certain TLRs (258).

Recognizing PAMPs (pathogen-associated molecular pattern= microbial components), TLR family is the crucial member of PRRs (pattern recognition receptors) in the innate immunity, which includes, NLRs (NOD-like receptors), RLRs (RIG-I-like receptors), ALRs (AIM2-like receptors) and CLR (C-type lectin receptors) (260, 261). TLRs also bind endogenous DAMPs (danger-associated molecular pattern= cellular components), released from cells due to damage or stress (260, 261). Since human TLRs are few, they must exert
extensive flexibility in order to respond to the huge number of pathogenic and harmful molecules (both PAMPs and DAMPs). Due to their critical roles in the immunity and survival of the host, TLRs activity should be highly regulated, well balanced and fine-tuned (262, 263). This complex and multilayered regulation is achieved by systemic regulation, signaling modulation (adapter molecules and negative regulations), co-receptors and homo- or heterodimerization, as well as crosstalk with other members of PRR family (262, 263). Activation of TLRs, dependent on the type of cell and stimuli, initiates specific anti-pathogenic immunological responses, resulting in production and secretion of pro-inflammatory chemokines, cytokines, interferons and antimicrobial agents, as well activating adaptive immune system (264).

TLRs are type I transmembrane receptors, with an N-terminal ligand-binding ectodomain, a single transmembrane helix, and a C-terminal cytoplasmic signaling domain (265, 266). The ectodomains are glycoproteins with leucine-rich-repeats (LRP), comprising of 550–800 amino acid residues, and are either extracellular or in intracellular endosomes (265, 266). The ectodomains usually consist 19-25 tandem LRP copies that are 19-25 amino acid long each, containing hydrophobic residues. A loop-connected a β-strand and an α-helix make each LPR that align in parallel and bind closely together by hydrogen-binding, forming a β sheet. This forces the solenoid-shaped LRP structure to form a curved configuration, where the hydrophobic residues point to the interior side, building a stable core (265, 266) (Figure 24A,B). The cytoplasmic signaling domains are named TIR domains (Toll/IL-1R/resistance), due to the shared homology with the IL-1R signaling domain (265, 266). TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 localize on the cell membrane while TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13 are present on intracellular compartments such as endoplasmic reticulum (ER), endosome, lysosome, or endolysosome (265, 266).
Figure 24. Schematic illustration of the Structure of Leucine-Rich Repeats (LRR) (265) and the cellular trafficking of TLRs (266). (A) Residues forming the β strand are highlighted in orange that form a hydrophobic core. (B) Ribbon diagram of TLR3. (C) Cellular trafficking pathways.

TLRs are produced in the ER and transferred to the Golgi, where they are processed and transported either to cell surface or to endoplasmic compartments. The ER-transmembrane proteins, UNC-93B, PRAT4A (also called CNPY3) and gp96 (also known as HSP90β1, Endoplasmin, GRP94 and TRA1) regulate the trafficking of TLRs from ER (266, 267) (Figure 24C). TLRs’ intracellular localization is suggested to be crucial for recognition and discrimination between self-nucleic acids and viral DNA/RNA, preventing TLRs from causing autoimmunity (267, 268). Interestingly, UNC-93B1 controls the excessive activity of TLR7 by activating TLR9, indicating that balance between TLR7 and TLR9 is a regulatory mechanism for autoimmunity (267, 268).

In association with TLR1 or TLR6, TLR2 recognizes lipoproteins, lipoteichoic acid, peptidoglycans, mannan, tGPI-mucin and zymosan, while TLR4 senses LPS, and TLR5 binds flagellin (268). TLR10 detects influenza A virus and contributes to TLR2 in sensing Listeria. (268) TLR3 detects double-stranded
RNA (dsRNA), self-RNA and small interfering RNA (siRNA), where TLR7 binds single-stranded RNA (ssRNA) and TLR8 recognizes both bacterial and viral RNA (268). TLR9 responds to DNAs and hemozoin. TLR13 detect bacterial 23S rRNA (268).

**Ligands of TLRs**

TLRs recognize a wide variety of microbial molecules, so called PAMPs. Interestingly, TLRs respond differentially to a type of PAMP dependent on the intensity of the stimuli (i.e. the concentration of the ligand). Homologous to the natural ligands, some synthetic ligands have been manufactured and widely used as verified and specific activators of certain TLRs. In addition, TLRs sense endogenous molecules and activate proper signals. Notably, some of the ligands signal through two different TLRs, which might be a biological characteristic or result of experimental error due to limitation in purification/anticontamination methodologies. Bearing this in mind, some of controversial endogenous ligands are highlighted in bold in the Table below, which contains a summary of TLR ligands (260, 269-273) (Table 5).
<table>
<thead>
<tr>
<th>TLRs</th>
<th>Microbial ligands</th>
<th>Endogenous ligands</th>
<th>Synthetic ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Triacyl lipoproteins</td>
<td>Unknown</td>
<td>Pam3CSK4</td>
</tr>
<tr>
<td>TLR2</td>
<td>Lipoproteins/lipopeptides, lipoteichoic acid, Glucuronoxylomannan, fimbria, peptidoglycan Porins, Glycoinositolphospholipids, Zymosan lipooarabinomannan, phospholipomannan, β-Glycan (fungus), tGPI-mutin (Trypanosoma parasit), Hemagglutinin</td>
<td><strong>HSP60, HSP70, HMGB1</strong>, endoplasmin, human cardiac myosin, Eosinophil-derived neurotoxin <strong>Hyaluronan</strong>, monosodium urate crystals, <strong>biglycan</strong>, SNAPIN</td>
<td>Pam2CSK4, Pam3CSK4, FSL-1</td>
</tr>
<tr>
<td>TLR3</td>
<td>Viral dsRNA</td>
<td>Self-mRNA</td>
<td>PolyI:C, polyA:U</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS, GPI anchors (protozoa), viral envelop proteins, mannan</td>
<td>Fibrinogen, heparin sulfate, α, β-defensin, α-crystallin A chain, endoplasmin, fetuin A, fibronectin EDA, surfactant protein, resistin, tenascin-C, serum amyloid A, <strong>saturated fatty acids</strong>, HSP22, <strong>HSP60, HSP70, HSP72</strong>, S100A4/A8/A9, uric acid, <strong>biglycan</strong>, <strong>HMGB1</strong>, <strong>Hyaluronan</strong>, monosodium urate crystals, <strong>oxPAPC</strong> (an oxidized phospholipid)</td>
<td>LipidA derivates</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR6</td>
<td>Diacyl lipoprotein, LTA, Zymosan</td>
<td>Oxidized LDL, Amyloid-B, versican</td>
<td>Pam2CSK4, FSL-1, Macrophage-activating lipopeptide 2</td>
</tr>
<tr>
<td>TLR7</td>
<td>Viral and bacterial ssRNA</td>
<td>Immune complexes, self-RNA, let-7b mTLR7: miRNA-21, miRNA-29a</td>
<td>Thiazoquinoline/imidazoquinolin compounds, synth. let-7b</td>
</tr>
<tr>
<td>TLR8</td>
<td>Viral and bacterial ssRNA</td>
<td>Immune complexes, self-RNA bTLR8: miRNA-21, miRNA-29a</td>
<td>Thiazoquinoline/imidazoquinolin compounds</td>
</tr>
<tr>
<td>TLR9</td>
<td>Viral and bacterial DNA</td>
<td>Chromatin IgG complexes, self-DNA</td>
<td>CpG (class A, B and C)</td>
</tr>
<tr>
<td>TLR10</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>TLR11</td>
<td>Profiling, Uropathogenic bacteria</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>TLR12</td>
<td>Profiling-like molecules</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>TLR13</td>
<td>Bacterial 23S rRNA</td>
<td>Unknown</td>
<td>23S rRNA derived oligoribonucleotide</td>
</tr>
</tbody>
</table>
**Signaling by TLRs**

TLRs are expressed on cell surface or cytoplasmic compartments as monomers, but dimerize upon stimulation, forming homo- or heterodimers (265, 266). TLR2 can signal as a homomer (274), but also as heterodimers with either TLR1 or TLR6, while TLR5 constitutes a homodimer, when stimulated. TLR4 builds a heterotetrameric (double heterodimers) complex with co-receptor MD2 (myeloid differentiation factor 2; also known as LY96). Recently, it was reported that TLR4 can signal through homodimerization in response to cationic lipids (275). In humans, TLR10 is suggested to form homodimers and heterodimers with TLR1 or TLR2. TLR7, TLR8 and TLR9 are produced as stable homodimers, but in order to signal, their TIR domains come into close proximity upon activation (266). Activation of these intracellular TLRs requires proteolytic cleavage, an action that may provide more safety against autoimmunity induced by recognition of self-DNA/RNA. Ligand binding causes a substantial conformational change in the dimer of TLRs (265, 266). TLR3, in contrast to other intracellular TLRs, is synthesized as inactive monomer that is cross-linked directly to another TLR3 by dsRNA and thereby activated (265, 266).

TLRs utilize different cytosolic TIR domain-containing adapters for downstream signal transduction, including MyD88 (myeloid differentiation factor 88), TIRAP (TIR-associated protein; known also as MAL), TRIF (TIR domain-containing adapter protein-inducing interferon β) and TRAM (TRIF-related adaptor molecule; known also as TICAM2) (268, 276). TLRs employ inhibitory adaptors as well, comprising SARM (sterile α- and armadillo-motif-containing protein), TAG (TRAM adaptor with Golgi dynamics (GOLD) domain) and BCAP (B cell adapter for PI3K) (268). All TLRs use MyD88 in their signaling except for TLR3, which recruits TRIF exclusively (268). TIRAP (Mal) participates in the downstream signaling of only TLR2, TLR4 and TLR9 by recruiting MyD88 and binding, via its lipid-binding domain, to PI(4,5)P₂ and PI(3)P on the plasma membrane and endosome, respectively (276). However, the role of TIRAP in TLR4 signaling is indispensable, while it only sensitizes TLR2 and TLR9 when the stimuli are low or normal. TRIF, recruited only by TLR3 and TLR4, activates an alternative downstream signaling, leading to robustly activation of IRF3 and weakly activation of NF-κB and thereby
subsequent induction of interferons and inflammatory cytokines (268). Thus, TLR signaling can be distinguished in two pathways, namely MyD88-dependent way that is essential for early NF-κB activation and TRIF-dependent way, which contributes to the late phase of NF-κB activation (268) (Figure 25).

Figure 25. Schematic illustration of TLR singling pathways (266).
MyD88-dependent signaling is initiated, upon TLR activation, by formation of Myddosome, catalyzed by TRIF (268, 269, 277). Myddosome is a hetero-complex of six MyD88 and four IRAK4 (IL-1 receptor-associated kinase) and two IRAK1 or IRAK2 molecules (IL-1R associated kinases; are serine/threonine kinases), binding together by their death domains (268, 269). Myddosome-activated IRAK4 phosphorylates and activates IRAK1 or IRAK2, which further undergoes autophosphorylation and release to engage TRAF6 (RING-domain E3 ubiquitin ligase). IRAK1 is suggested to be essential in early NF-κB activation, while IRAK2 is important for maintained TLR signaling (268, 269). TRAF6 together with E2 ubiquitin enzymes, UBC13 and UBV1A, catalyzes K63-linked polyubiquitin-chains formation on TRAF6, resulting in activation of the regulatory subunits TAB2, TAB2 and TAB3 that activate TAK1 by forming a complex with it (268). Further, TAK1 via ubiquitin chains binds to IKK complex of catalytic subunits IKKα, IKKβ and regulatory NEMO, resulting in phosphorylation and activation of IKKβ. This permits the phosphorylation and degradation of IkBα, freeing NF-κB to translocate into nucleus and induce gene expression (268, 269). In addition, TAK1 triggers MAPK signaling by inducing ERK1/2, JNK and P38 that result in mRNA stabilization or activation of AP-1 transcription factor family to control inflammatory responses. In parallel, TRAF6 activation results in IRF5 and IRF7 activation, which contributes to production of proinflammatory molecules in a NF-κB dependent way (268, 269). In TLR2 and TLR4 signaling, TRAF6 can also translocate to mitochondria and interact with ECSIT (evolutionarily conserved signaling intermediate in Toll pathways), inducing its ubiquitination and enhanced subsequent ROS (Reactive oxygen species) production (268). Notably, TAK1 is suggested to exhibit a cell type-specific role, while TAB family proteins appear to compensate for each other in TLR signaling (268) (Figure 25 and 26).

TRIF-dependent signaling is triggered directly, upon activation of TLR3 and indirectly via TRAM by stimulation of TLR4. Interestingly, TRAM-bridged induction of TRIF requires internalization of TLR4 to the endosome, which is dependent on the presence of LPS-attached co-receptor CD14 (268, 269). TRIF recruits both TRAF6 and TRAF3 independently, where ubiquitin-activated TRAF6 induces RIP1 (receptor-interacting protein 1) kinase, and
polyubiquinated TRAF3 activates non-canonical IKKs, IKKi (same as IKKe) and TBK1 (TANK-binding kinase 1) (268, 277). TRAF6, via adapter protein TRADD (TNFR-1-associated death domain), activates Pellino-1 (E3 ubiquitin ligase), which induces RIP1 by ubiquitination. RIP1 interacts and induces TAK1 complex, resulting in activation of MAPK and NF-κB and thus inflammatory cytokines production (268, 277). TRAF3, via IKKi and TBK1 activates Pellino-1, which binds to DEAF1 (deformed epidermal autoregulatory factor 1) transcription factor and promotes IRF3 dimer to bind to IFNβ promotor (268, 277). In parallel, PtdIns5P (phosphatidylinositol-5-phosphate) forms a complex with IRF3 and IKKi/TBK1, leading to phosphorylation, activation and dimerization of IRF3, which thereafter translocates into nucleus and induces gene expression in association with β-catenin and CBP (CREB binding protein) in a HDAC6 (histone deacetylase) dependent way (268, 277) (Figure 26).

Figure 26. Schematic illustration of Myddosome and TRIF signaling pathways (268).
TLR7 and TLR9 signal in two different ways: A MyD88-NF-κB dependent pathway that leads to induction of inflammatory cytokine, and a MyD88-IRF7 dependent pathway, which results in production of type I IFNs (261, 268). Interestingly, upon activation, TLR9 is transported initially to VAMP3-positive early endosome, where it engages MyD88-NF-κB pathway and later on, traffics under the control of AP3 to LAMP2-positive lysosome-related organelles (LROs) to activate MyD88-IRF7 pathway (261, 268). Upon stimulation of TLR7 and TLR9, a Myddosome is formed and recruits TRAF6 that after activation by ubiquitination triggers either TAK1-NF-κB pathway or IRF5 dependent gene expression (261, 268). TLR7 and TLR9 achieve activation of IRF7 by Myddosome, TRAF6, TRAF3, IKKα, OPNi (a precursor of osteopontin) and DOCK2 (dedicator of cytokinesis 2) through formation of a complex, where antiviral protein Viperin activates IRAK1 by ubiquitination, which subsequently together with IKKα phosphorylate and activate IRF7. IRF7 then dissociates and translocates into nucleus (261, 268) (Figure 27).

Figure 27. Schematic illustration of intracellular TLR trafficking and signaling (268).
It is suggested in the literature that tyrosine phosphorylation of the TIR domains plays a critical role in the recruitment of adapter molecules and that PI3K participation in the TLR signaling complexes is important (277, 278). Upon activation and dimerization of TLR3, phosphorylation of two tyrosine residues, Tyr759 and Tyr858 by ErbB1 (epidermal growth factor receptor), Src and Btk (Burton’s tyrosine kinase) is pivotal for the recruitment of TRIF and its signaling (277, 278). Furthermore, PI3K-Akt signaling is necessary for full activation of IRF3, as inhibition of PI3K-Akt abolishes the transcriptional activity of IRF3 as well as NF-κB, but not the translocation into nucleus (277). Similarly, phosphorylation of tyrosine residues of TLR2, TLR4, TLR5, TLR8 and TLR9 is necessary for TIR adapter proteins recruitment and their signal induction (277) (Table 6). PI3K-Akt pathway plays various roles in signaling of different TLR, dependent on the cell type. It suppresses TLR signaling in dendritic cells, macrophages and monocyte, but promotes TLR-induced immune response in mast cells (278).

**Table 6. A summary of tyrosine residues and kinases involved in TLR signaling.**

<table>
<thead>
<tr>
<th>TLRs</th>
<th>Required tyrosine residues</th>
<th>Involved tyrosine kinases</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>Tyr616, Tyr761</td>
<td>Btk, Fyn</td>
</tr>
<tr>
<td>TLR3</td>
<td>Tyr759, Tyr858</td>
<td>ErbB1, Btk, Src</td>
</tr>
<tr>
<td>TLR4</td>
<td>Tyr674, Tyr680</td>
<td>Btk, Lyn, Hck, Syk</td>
</tr>
<tr>
<td>TLR5</td>
<td>Tyr798</td>
<td>Unknown</td>
</tr>
<tr>
<td>TLR8</td>
<td>Tyr898, Tyr904</td>
<td>Btk</td>
</tr>
<tr>
<td>TLR9</td>
<td>Tyr1048</td>
<td>Btk, Src, Syc</td>
</tr>
</tbody>
</table>
Regulation of TLR signaling

Regulation of TLR signaling is crucial to protect against excessive cellular and tissue damage and tumor cell growth, and to prevent autoimmunity. There are several modulatory mechanisms functioning on multiple levels, including regulation of receptors, adapters, Myddosome, signaling mediators, transcription factors, post-transcription factors and post-translation products (279). Dimerization of TLRs, utilization of co-receptors and decoy receptors (e.g., soluble TLRs, CD14 and ST2 and membrane-bound ST2L) are some ways to prevent overactivation of TLRs (279). Crosstalk between TLRs is another way to regulate TLRs signaling (280). Activation of MyD88- and TRIF-dependent signaling pathways is balanced through TRAF3, which promotes TRIF pathway, but inhibits MyD88 pathway (268, 280). NRDP-1 (E3 ubiquitin ligase) that degrades MyD88, augments TBK1 activity, and favors IFN induction over inflammatory cytokines (268). In addition, cytoplasmic tyrosine phosphatase SHP-1 (SH2-containing protein tyrosine phosphatase 1) promotes TRIF-dependent IFN production and inhibits NF-κB mediated cytokine production, while SHP-2 inhibits TRIF-dependent signaling pathway (277). MHC class II molecules, located in endosomes, maintain Btk activation via the costimulatory molecule CD40 and consequently promote the activation of both MyD88 and TRIF signaling (268). As downstream regulators, ST2825, SOCS1 and Cbl-b suppress MyD88-dependent pathway by inhibiting the binding of MyD88 to other downstream adapters (281) (Figure 28). Likewise, SARM and TAG prevent TRIF association to other adaptors (281) (Figure 29). DUBA and SOCS3 diminish the activation of TRAF3, while TRAF6 activation is negatively regulated by A20, CLYD, SHP, TANK, TRIM38 and USP4. A20 and TRIM30α inhibit TAK1 signaling (281). In addition, ATF3, Bcl-3, IkBNS, Nurri1 and PDLIM2 limit the transcriptional activity of NF-κB, as Pin1 and RAUL suppress IRF3 activity (281). IRF4 inhibit the activity of IRF5 by competing to bind to MyD88. Moreover, CEACAM-1, PTP-PEST and Tir regulate the PI3K-Akt pathway (277, 281). While autophagy and crosstalk between TLRs and with other PRRs modulate TLR signaling (279-281), miRNAs fine-tune the extremely complex system of TLR signaling (281, 282) (Figure 30).
**Figure 28.** Schematic illustration of negative regulation of MyD88-dependent pathway in TLR signaling (281).
**Figure 29.** Schematic illustration of negative regulation of TRIF-dependent pathway in TLR signaling (281).
Figure 30. Schematic illustration of the fine-tuning of TLR signaling by miRNA (282).
**Co-receptors of TLRs**

Membrane-anchored or soluble molecules often assist TLRs in ligand binding to ensure correct interactions. These molecules are function as co-receptors and assist TLR-function in various ways, like capturing the dispersed ligands in the extracellular space and concentrating and introducing them to TLRs. They can also delineate the specificity and/or enhance the affinity of TLRs' ligand binding or complement TLR signaling through activating parallel signals. In addition, co-receptors can facilitate the internalization and intracellular trafficking of TLRs' receptor-ligand complexes. TLRs co-receptors include CD14, CD36, HMGB1 (high-mobility group box 1), LBP (LPS-binding protein), LL-37 (an amphipathic peptide) and MD2 (268, 283).

**Toll-like receptors (TLRs) and bone**

The bone tissue is often affected by infection-induced and sterile inflammatory diseases as well as by malignant tumors. The result is usually an osteolytic lesion, but sometimes the outcome is a sclerotic reaction. Bone tissue reacts also to foreign materials such as wear particle from orthopedic implants. In osteomyelitis, the bone is infected and colonized by bacteria. Osteotropic inflammatory cytokine could be the link to activation of bone resorbing osteoclasts, but the involvement of TLRs of the innate immune system in recognizing bacterial components and foreign materials could be the key factor in inflammation-induced bone destruction, since LPS was found to induce bone resorption (284) and since TLR4 is a critical receptor for LPS (285). In addition, it was reported that LPS could induce osteoclast formation directly in committed pre-osteoclasts, but inhibited osteoclastogenesis in OCPs (286).

Bone cells express TLRs and respond to various stimuli by PAMPs and DAMPs (287). Osteoclast precursors express TLR1 to TLR10, but mature osteoclasts express only TLR2 and TLR4 (287). The outcome of TLR activation in bone tissue is predominantly osteolytic. Yet, TLR (TLR1-TLR6 and TLR9) activation in OCPs abolishes osteoclastogenesis induced by RANKL. Interestingly, activation of TLRs (TLR1-TLR6 and TLR9) on committed pre-osteoclasts results
in enhanced osteoclast formation, and stimulation of TLR2 and TLR4 in mature osteoclasts promote the survival of these cells (287-291). Osteoblasts express TLRs as well, and in response to TLR activation express inflammatory cytokines and RANKL, promoting osteoclast formation (287).

**Aims**

Inflammatory processes in the vicinity of the skeleton usually affect the remodeling of the nearby bone tissue. Conditions like periodontitis, peri-implantitis, osteomyelitis, rheumatoid arthritis and loosened orthopedic prosthesis are examples of such phenomenon. The situation is similar to the reactions in bone induced by malignant tumors. The clinical manifestation is often osteolytic (loss of bone), but sometimes the reaction is sclerotic. Since bone tissue interacts with other organs or systems and contributes to their homeostasis and regulation, and the susceptibility and severity of diseases vary between individuals, a profounder understanding of how pathogens affect bone remodeling is likely to aid identifying new strategies for the management of such pathological conditions. Because Toll-like receptors are the key initiators of responses to pathogens and are expressed in bone tissue, we aimed in this project to elucidate the importance of different TLRs for inflammation induced bone loss.

Since only TLR4 in bone was extensively studied and the role of TLR2, which recognizes pathogenic bacteria like *P. gingivalis* and *S. aureus*, and TLR5 as the only protein-recognizing receptor in bone was elusive, the specific purposes of this thesis were to explore:

- The role of TLR2 in bone when activated by major pathogens like *P. gingivalis* and *S. aureus*
- Screening for the role of other TLRs in bone, which led to the identification of a novel role of TLR5 in bone
- Identification and description of a new effect of TLR activation *in vivo*, namely bone formation
Methodological considerations

Material and methods used for this thesis are described in details in the Materials and Methods section of each paper. Here follows an overview with a general discussion of the important aspects of the employed methods and materials.

**TLR agonists**

We used synthetic ligands for TLR2 as positive controls in our experimental protocols. Since the specificity and bioactivity of these synthetic ligands are verified (292-294), we included them to ensure and compare our observations by bacterial TLR2 ligands. Pam2CSK4 and Pam3CSK4 activate TLR2/TLR6 and TLR2/TLR1 heterodimers respectively, which enabled us to compare the activity of both dimers to the induction by LPS from *P. gingivalis* (LPS *P.g.*) and *S. aureus*. As mentioned in the beginning of this thesis, *P. gingivalis* is a major pathogen in periodontitis and is associated with RA. *S. aureus* is a potent and invasive bacterium causing a wide range of infections. Several of these infections affect bone tissue, such as septic arthritis, osteomyelitis and infected orthopedic implants. LPS *P.g.* is controversial in regards of whether activating TLR2 or TLR4. LPS *P.g.* structure varies dependent on the environmental conditions (so called heterogeneity) and ultra-pure LPS *P.g.* with no lipoprotein “contamination” that consists of S-(2,3-dihydroxypropyl)-cysteine with three fatty-acid residues, can activate TLR4 (295). Although, it is widely accepted that LPS *P.g.* is a TLR2 agonist, the use of TLR2 knockout cells and tissue in the studies included in the present thesis exclude the involvement of TLR4 in the studied effects.

Flagellin, a 28-80 kDa protein depending on the bacterial species, is the major component of the flagella of motile bacteria, responsible for locomotion. It is the only protein PAMP, which is the target for TLR5 (296). Flagellated bacteria are present in the periodontal pocket and contribute to the pathogenesis of periodontitis and acute necrotizing ulcerative gingivitis (21, 297) and septic arthritis (298, 299). TLR5 is also involved in RA (300, 301). We used two different ultra-pure flagellins, from *Salmonella typhimurium* (FLA-S.t.) and
*Bacillus subtilis*, to compare the activity and minimize the risk of endotoxin contamination (302), although, the only way to ensure the exclusiveness of TLR5 activation was to use TLR5 deficient cells and tissue in the presence of flagellin or LPS.

**Animals**
The widespread use of animals in research is due to the ethically and difficulty issues with humans as subjects when researchers use “Trial and Error” approach. Mice are favored and mostly used experimental animals because of ease of use and handle of these animals. They are small, cheap to keep and breed fast in a short time, which make them well situated for genetic modification. Despite the transferability issue of research from animals to humans, animals provide the proper environment with biological and optimal tissue interactions and complexity. Although, there is certainly a slight difference in biological response to various stimuli between diverse wild-type strains, the discrepancy is not considered as a major issue. Our use of CSA-mice and C57-BL6 mice was simply due to availability and matched knockout littermates (TLR2 and TLR5). The knockout mice (both TLR2 and TLR5) do not have any known bone phenotype.

The local ethics committees at Umeå University, Umeå and University of Gothenburg, Gothenburg, Sweden approved all animal care and experiments, which were conducted in accordance with the accepted and appropriate standards of humane animal care and use.

**Gene and protein expression analyses**
To quantify the expression of a certain gene of interest, we measured the amount of copies of its mRNA transcript in a given sample. In order to do that, the isolated mRNAs was reversely transcribed to cDNA (complementary DNA) and analyzed by a PCR (polymerase chain reaction) method like semi- quantitative or quantitative PCR with a thermal cycler, which includes three steps: Separation of DNA double strands, ligation of primers with template DNA and polymerization (elongation).
Real-time quantitative PCR (q-PCR) is a sensitive method for quantification of gene expression. It is called real-time because the detection of amplified DNA occurs during the reaction progress. cDNA is mixed, in a buffering solution containing thermo-stable DNA polymerase, with specifically designed primers (forward and reverse; oligonucleotides) and probe complementary to a specific sequence of the target gene. The probe contains fluorescent reporter on one end and quenching dye on the other end to prevent emission from the reporter. During, polymerization (elongation) of the PCR product the hybridized probe is degraded by the polymerase and the reporter dye is cleaved off by the polymerase (released from the quencher) and starts to emit fluorescence, allowing the detector to measure the intensity. FAM-labeled dyes have a non-fluorescent quencher compared to TAMRA, which reduce the background noise (303, 304).

q-PCR-based quantification of gene expression can be utilized by two methods, the absolute quantification that counts the exact target DNA template number by comparing it to DNA standards in a calibration curve, or the relative quantification by using an internal reference gene (Housekeeping) to calculate fold-changes in mRNA expression of the target gene. The measurement of fluorescence is made over a given threshold to eliminate the background noise. Quantification cycle (Cq, also called threshold cycle) is the number of cycles when the fluorescence exceeds the threshold, which is plotted against the standard curve (to determine the efficiency of a primer-template combination, assessed by serial dilutions of DNA template in a titration experiment) on a logarithmic scale. The relative quantification method is easier, less time consuming and better to use since standards with known concentrations is not required and the reference gene can be any transcript with a known sequence. In addition, DNA-based calibration curve is only subjected to PCR steps (unlike mRNA samples that first reversely transcribed), increasing the potential for variability in the amplification result, which may be different and incomparable to the amplification from the unknown samples. Thus, the starting cDNA amount in the samples and the use of a reference gene to normalize with are important, in order to control the amplification variability (303-305). q-PCR can also be used with other techniques like non-specific DNA binding fluorochromes (e.g., SYBR Green) and hybridization probes. It is a common and accepted practice (in the
bone research field) to normalize q-PCR amplification to either β-actin, GAPDH or 18S rRNA as reference genes, but the expression of these genes may fluctuate between samples. This may raise the questioning of the accuracy and reliability of data with regulation of two fold or less (both upregulation and downregulation) (306). Optimally, the reference gene should be validated in the same samples. Furthermore, there are some other considerations regarding the potential errors in quantification of q-PCR readouts, including the quality of extracted RNA (i.e. how much of the total RNA is reversely transcribe), the fraction of mRNA in the total purified RNA and contamination of genomic DNA or alien molecules in the cDNA (305).

In addition to equal sample size and DNAse treatment of prepared mRNA, we always include a pooled sample of mRNAs of every experimental group with no reverse transcriptase to ensure that no amplification is registered due to the presence of genomic DNA for each analyzed gene (307).

In all the in vitro and ex vivo experiments, the reference genes used did not vary between control and experimental samples. In the last in vivo study of paper IV, the cDNA was synthesized using the same volume instead of equal amount of RNA per sample. In order to normalize for this discrepancy the expression of genes were divided by the total RNA amount of the sample. Due to the large differences in cell number and cell types in the skull bones between controls and stimulated mice, which resulted in great differences in amount of extracted RNA, the expression of reference genes varied and we did not succeed to find a reference gene that was not changed when related to RNA amount. Genes such as Tnfsf11 (encoding RANKL), Acp5 (encoding TRAP), Ctsk (encoding cathepsin K), Tnfrsf11b (encoding OPG), Calcr (encoding calcitonin receptor), c-Fos and Nfatc1 were very highly expressed in the stimulated samples and therefore still upregulated after normalizing with the fluctuating reference genes. However, genes such as Alpl (or Akp1; encoding ALP), ColIa1 (encoding collagen type I), Bglap (encoding osteocalcin), Sp7 (encoding osterix) and Runx2 were only upregulated in restricted areas of the whole skull bones and normalizing these genes with reference genes implied that their expression was downregulated (Paper IV). We, therefore, “normalized” their expression by relating their expression to each calvarium (i.e. the amount of RNA input in each cDNA
reaction). This is not optimal, of course, and we need to assess their expression levels in the region of interest by harvesting RNA using other techniques like laser capture microscopy and/or by confirming their expression using immunohistochemical techniques.

Semi-quantitative reverse transcription PCR (RT-PCR) visualizes PCR products on a gel, which indicates the changes in the expression of a targeted gene. For most of the genes analyzed, we confirmed the identity of the products by sequence analyses, a quality control that cannot be made using the quantitative real-time PCR technique. Semi-quantitative PCR, however, is a time-consuming method and its quantification is less reliable.

Gene expression analyses at mRNA level indicate a biological response, but do not corroborate the actual effect because not all mRNA amount is translated into actual protein and indeed, whether the expressed protein is biologically active. With this in mind, protein expression analyses should accompany mRNA expression analyses (307). Protein expression can be analyzed by semi-quantitative gel-based Western blot or more sensitively and quantitatively by ELISA (enzyme-linked immunosorbent assay), which can utilize either radioactive labeling or antibody tagging (308). There are other techniques to assess gene expression like micro-arrays and reporter gene assays.

ELISA utilizes immobilization of the proteins on a specific surface and enzyme-conjugated antibodies that bind to the protein. Addition of enzyme specific substrate carrying a chromophore results in the release of the chromophore in proportion to the amount of bound antibodies and the chromophore can be measured by a spectrophotometer. The concentration of the chromophore reflects the amount of antigen, which can be quantified using standard curve method. ELISA is specific, precise and quantitative method that is easy and fast to use.

Luciferase reporter gene assay
Several different reporter gene assays can quantify the transcriptional activity (both increased and decreased) of a target gene, which can serve as an indicator of intracellular signal transductions. Reporter gene assays have a high
sensitivity. The reporter gene, which can be a luciferase or a FP (fluorescent protein), is expressed under the control of a TATA-box coupled to a specific transcriptional response element (TRE) of a transcription factor of interest. The reporter gene-containing construct is transduced into a cell by a viral vector (in our case lentivirus) or by liposome complex for plasmids with reporter construct, and once the targeted transcription factor starts its activity the reporter gene (luciferase) starts to be expressed. The expression of luciferase is correlated to the activity of the investigated transcriptional factor. To assess the transcriptional activity, we measured the luciferase expression in the cell by a luminometer. Reporter gene assays have a wide range of applications (309, 310), but we used this method to assess the NF-κB activity.

**Isolation of cells**

Parietal (calvarial) cells were isolated using a time sequential collagenase digestion technique that results in isolation of calcitonin (CT) responsive cells (very few) in the first three digestions, mix of CT-positive cells and osteoblast-like cells responsive to PTH in digestions 4-6 and mainly PTHR-positive osteoblast-like cells in the last digestions. The last fractions were then cultured and after 24 h, the cells were washed and further cultured for 48 h. We considered and used these cells as osteoblasts in the experiments, despite the fact that they were confirmed contaminated with cells of osteoclastic origin (311). Due to difficulty of further purifying osteoblasts (e.g., no reliable surface marker for FACS sorting available) and the probable low impact of the contamination in the total response, it is accepted to use these osteoblast-rich cultures as osteoblasts. In favor of this assumption, we noted a completely different outcome when cells from all 10 fractions together were cultured and stimulated (Paper I and II).

Osteoclast precursors can be isolated from bone marrow in two different ways after lysing the erythrocytes, collection of non-adhering cells (after 2 h) or adhering cells on CORNING® (optically-clear non-treated for cell attachment polystyrene) plate (after 48 h in presence of M-CSF) (145, 312). We chose the latter method because the obtained cells were selectively isolated (due to the plastic in the used Corning plates) and expanded by M-CSF. Compared to non-
adherent precursors, these pure pre-osteoclasts are not contaminated with other non-adherent hematopoietic cells.

The co-culture of parietal osteoblasts with BMM (bone marrow macrophages) is a commonly used method to investigate interaction between these two cell types. We chose a more natural co-culture system with cells of osteoblasts and pre-osteoclasts isolated from parietal bones. These cells are similarly pre-conditioned in the context of micro-milieu.

**Bone organ cultures**

Bone organ culture is the optimal *in vitro/ex vivo* system to study interactions between the bone tissue and its cells, when affected by an exogenous stimulus. Culture of bone organ like parietal bone provides the 3-D microenvironment suitable for cell interactions without influence from inflammatory infiltrates or endocrine factors. We used parietal bones because our primary focus was the study of periosteal bone.

**Cell cultures**

To study the effect on a specific isolated cell type, *in vitro* cell culture is an easy and preferable method. This allows the exclusion of other cofactors that may interfere or somehow modify the outcome. *In vitro* cell cultures are best suitable for investigating intracellular signaling, gene and protein expressions. Immortalized cell lines or primary cells are usually used for cell cultures. Cell lines (often from tumors) are favored when the primary cells are hard to acquire or purify like osteocytes, but due to numerous passages of proliferation, they might lose, change or develop new characteristics. With this in mind, we chose to use primary cells in our cultures, although we had access to matched cell lines.
Enzyme-cytochemistry, enzyme-histochemistry and immunohistochemistry

Visualizing the morphology of cells and tissues is a very nice, informative and corroborative way to support the molecular observations. Enzyme-cytochemistry and enzyme-histochemistry utilize chemical dye to stain cytosolic enzymes in cell cultures and tissues, respectively, while immunohistochemistry uses antibody conjugates to stain tissues. TRAP and cathepsin K staining are frequently used to demonstrate osteoclasts, whereas ALP is often used to identify osteoblasts and Alizarin red to show calcium containing mineralized bone matrix.

Flow cytometry

Flow cytometry is a biophysical technique that utilizes excitation by laser and fluorochromes emission for counting and sorting cells and detection of biomarkers. Suspension of cells in a fluid stream passes through a FACS machine (fluorescence-activated cell sorter), in which antibody-conjugated fluorochromes emit fluorescence upon excitation and registered by detectors. Fluorochromes can either be coupled to surface epitopes or conjugated to antibodies to identify intracellular molecules. FACS can perform simultaneous multi-parametric analysis very fast and with side and forward light scatter, the relative size and granularity of cells can be distinguished. However, scattered light from debris and/or intracellular structures can interfere with emitted fluorescence and give false positive results. This phenomenon is called autofluorescence (313). We used FACS to verify protein expression of TLR5 on the cell surface, which was a confirmation of gene expression analyses.

Microcomputed tomography (µCT)

µCT analysis provides a three-dimensional imaging with a very high resolution, enabling the detailed study of the microarchitecture of bone by visualization and measurement. µCT facilitates the study of very large specimen (e.g., whole calvarium, tibia or vertebrae). The 3-D image is a mathematical reconstruction of the X-ray image. The bone sample is placed on rotator between the X-ray
source and the detector rotate during the radiation. It is a very powerful tool, especially in the field of bone research, but it is expensive and time consuming.

**Statistics**
In the parametric methods of testing data, the statistical method is estimating the probability of whether an observed effect is due to randomness, which requires normal distribution of data (normality). When the experimental setup contains several groups (more than two) with multiple treatments (replicates), the statistical method of choice is one way ANOVA (analysis of variance), which enables the pairwise comparison between groups or versus-control comparison. ANOVA can only be applied when the distribution is normal and there is homoscedasticity of variance (homogeneity of variance). There are several post hoc test available for this method, but the Holm-Sidak’s test is more powerful than other tests and is applicable for pairwise and versus-control comparison. For comparison of two groups with multiple replicates, paired t-test is appropriate, but if the normality test fails, Mann-Whitney Rank Sum test could be performed (314).
Results and discussion

In the result section of this thesis, TLRs means TLR2 and TLR5.

*Enhanced bone resorption by TLR activation ex vivo*

Stimulation of TLR2 by LPS *P.g., S. aureus* and its synthetic ligands (*i.e.* Pam2 and Pam3), as well as TLR5 by flagellin, resulted in enhanced bone resorption in a time- and concentration-dependent manner in organ-cultured parietal bones. The effect was assessed by analyzing the release of mineral and of matrix degradation fragments, and was associated with enhanced osteoclast number and expression of osteoclastic genes, including *Acp5* (encoding TRAP), *Ctsk* (encoding cathepsin K) and the early response transcription factor *c-Fos*. Expression of other genes related to osteoclastogenesis, such as *Csf1* (encoding M-CSF), *Csir* (encoding c-Fms), *Oscar* and *Tnfrsf11a* (encoding RANK) was also increased. Furthermore, the addition of the bisphosphonate zoledronic acid abolished TLRs-induced bone resorption. These findings show that TLR2 and TLR5 induced bone resorption through enhancing osteoclast formation and activation.

The time- and concentration-dependent increase of bone resorption parameters shows that the observed response is a biological effect caused by activation of TLRs. In addition, enhanced expression of osteoclast-related genes indicates that increased bone resorption is due to enhanced osteoclast differentiation. The inhibition of TLR-induced bone resorption by bisphosphonate proves that osteoclasts were mediating the effect. The degree of *ex vivo* induction of bone resorption by TLR2 and TLR5 is comparable with bone resorption induced by RANKL and TLR4 agonist LPS from *E. coli* (LPS *E.c.*).

*TLR-induced bone resorption is due to enhanced RANKL/OPG ratio*

Activation of TLR2 and TLR5 by their respective ligands resulted in enhanced expression of *Tnfsf11* in a time- and concentration-dependent way in the parietal bones. This augmentation was accompanied by increased protein expression of RANKL in these bones. Interestingly, stimulation of TLR2 by its ligands did not
alter the expression of Tnfrsf11b, while activation of TLR5 did decrease Tnfrsf11b expression time- and concentration dependently. These observations were made at both mRNA and protein levels. Adding exogenous OPG diminished bone resorption and cathepsin K expression induced by TLR2 and TLR5 agonists while Tnfsf11 expression was not affected. These observations demonstrate the crucial role of enhanced RANKL in TLRs-induced bone resorption.

Stimulation of TLR2 and TLR5 in co-cultures of parietal cells (i.e. periosteal/endosteal osteoblasts and osteoclast precursors) led to osteoclast formation and enhanced osteoclast markers. Challenging the TLR stimulated cells with exogenous OPG abolished osteoclast formation, further demonstrating the role of RANKL in TLR-dependent osteoclastogenesis.

It is well known that RANKL is essential in osteoclast formation and activation, but the direct activation of TLRs in pre-osteoclasts has also been shown to induce osteoclastogenesis independent of RANKL (287), an effect we also observed by TLR2 and TLR5 activation. Notably, the RANKL-independent effect of TLRs activation on osteoclast formation is only seen in isolated committed (stimulated with RANKL for at least 24 h) osteoclast precursors in vitro. This effect has been shown to be mediated by TNF-α in response to TLR4-activation (287) and by NFATc1 with partial aid from IL-6 and TNF-α in reaction to TLR2-activation (291, 315). We managed to confirm that osteoclastogenesis induced by TLR4 ligand, LPS E.C., was TNF-α mediated using anti-TNF-α, but did not experience any effect on TLR2-mediated osteoclast formation in the parietal bones by neutralizing IL-6 and TNF-α (data not shown).

Conversely, stimulation of TLRs on early osteoclast progenitors (e.g., BMM) does not stimulate osteoclastogenesis, but inhibits RANKL-induced osteoclast formation in these cells (287, 288). Remarkably, unlike TLR2 and TLR4, we did not observe any inhibitory effect by TLR5-activation on RANKL-stimulated osteoclast formation in BMM and the absence of effect was not due to lack of TLR5 expression on the cell surface of BMM. Although, it has been reported that activation of TLR5 inhibits osteoclastogenesis induced by RANKL (288), this discrepancy could be explained by the use of different agonist (FlaB) versus flagellin. However, the explicit specificity of FlaB to TLR5 was not verified.
Moreover, activation of TLR2 and TLR5 did not suppress RANKL–induced bone resorption in organ-cultured bones, nor did TLR5 potentiate it. These results show that stimulation of TLR2 or TLR5 in bone tissue causes bone resorption \textit{ex vivo} by augmenting RANKL/OPG ratio.

\textit{Activation of TLRs induces bone resorption independently of cytokines and prostaglandins}\n
Stimulation of TLR2 and TLR5 by their respective ligands enhanced the expression of pro-inflammatory cytokines, including IL-1β, IL-6, IL-11, LIF, OSM and TNF-α and prostaglandin E\textsubscript{2}. These cytokines and prostaglandin are potent stimulators of osteoclastogenesis that could mediate the osteoclastogenic effects by TLRs. To investigate this possibility, we neutralized these cytokines, using specific antibodies and observed no alteration in TLRs induced bone resorption, nor in \textit{Tnfsf11} (encoding RANKL) expression. The efficiency of used antibody concentrations was confirmed in separate experiments and to exclude the possibility of simultaneous upregulation of several cytokines, we added the antibodies all together in a mixture. Thus, no notable change or reduction could be registered. To inhibit prostaglandin biosynthesis, we added indomethacin to TLRs-stimulated parietal bones, which had no effect on bone resorption, but partially reduced the expression \textit{Tnfsf11}. These experiments demonstrate that bone resorption and \textit{Tnfsf11} expression induced by TLR2 and TLR5 activation is independent of cytokines expression, and that prostaglandins have no significant effect on TLRs-mediated bone resorption despite the partially altered \textit{Tnfsf11} expression.

\textit{TLR-activation enhances RANKL/OPG ratio in osteoblasts independently of cytokines and prostaglandin}\n
In accordance with the observations in parietal bones, TLR2 and TLR5 agonists upregulated \textit{Tnfsf11} expression significantly in a time- and concentration-dependent way in isolated osteoblast-enriched cell cultures \textit{in vitro}. A similar pattern to that in parietal bones in regulation of \textit{Tnfrsf11b} expression was noted, where TLR5 stimulation decreased the expression \textit{Tnfrsf11b} time- and
concentration dependently and TLR2 activation had no effect on Tnfrsf11b. On the contrary, it was reported that TLR2 activation by its synthetic ligands in osteoblast-BMM co-culture system resulted in decreased OPG expression (291). The contradicting observations might be due to mechanistic dissimilarity in the employed procedures, where we used isolated enriched osteoblasts cultures and parietal all-cells co-culture, while they used cells from different origins (i.e. osteoblasts and BMM). In addition, we assessed the mRNA expression of OPG in the isolated cells, but they measured the protein in the culture media. However, we feel confident to trust our data, since we obtained similar response by use of six different ligands and it corresponds to the observations in the organ-cultured parietal bones.

As expected, challenging osteoblasts with TLR2 and TLR5 agonists significantly enhanced the expression of abovementioned cytokines (with the exception of IL-11 and OSM in TLR5 activation) and prostaglandins, but neutralizing these cytokines by an antibody-cocktail did not affect Tnfsf11 expression. Interestingly, blocking prostaglandin biosynthesis by indomethacin completely abolished the TLR2-induced expression of Tnfsf11 in these cells, but only partially reduced Tnfsf11 expression induced by TLR5.

Despite the complete inhibition of RANKL in isolated osteoblasts, indomethacin failed to inhibit osteoclast formation in our co-culture system and committed pre-osteoclasts (RANKL-primed BMM) cultures, which also was supported by independent observations from another group of researchers (291). In addition, indomethacin was unable to suppress the bone resorption as mentioned earlier.

Noteworthy, activation of TLR2 and TLR5 auto-upregulate their own expression in the isolated osteoblasts. A possible mechanism was suggested to be via TNF-α, as reported in human gingival fibroblasts (316), but we did not investigate this possibility.
The observed effects are indeed due to activation of TLR2 and TLR5, respectively.

We used several agonists to activate TLR2 in addition to LPS *P.g.*, including *S. aureus*, HKLM (heat-killed *Listeria monocytogenes*) and especially the specific synthetic TLR2 ligands: FSL-1, Pam2 and Pam3, as positive controls. The similar and comparable results by all these ligands indicated that LPS *P.g.* activated TLR2, but we could not rule out the possibility of activating TLR4 by *P. gingivalis* LPS. Thus, we used *Tlr2*⁻/⁻ (knockout) osteoblasts, which proved that the observed upregulation in *Tnfsf11* expression was critically dependent on TLR activation.

Likewise, we included two types of flagellin in our experimental protocols to activate TLR5 and obtained comparable results. We were, however, unable to exclude the risk of endotoxin (LPS) contamination in flagellin preparations and thereby, the possibility that the effects were due to a TLR4 mediated response. Using *Tlr5* deficient osteoblasts and parietal bones demonstrated that the induction of bone resorption and upregulation of osteoclast-related genes by flagellins is explicitly mediated through TLR5.

In vivo activation of TLR2 and TLR5 results in increased osteoclast formation and significant bone loss.

To investigate whether activation of TLR2 or TLR5 results in effects in vivo, corresponding to the in vitro/ex vivo observations, we used an in vivo model of periosteal bone resorption. We injected 5 week-old mice with saline, TLR2 agonists or TLR5 agonist subcutaneously on the top of the skull bone in both wild type mice and TLR2- or TLR5 null mice. After euthanizing the animals, the skull bones were collected for radiological, histological and gene expression examinations.

In vivo stimulation of TLR2 and TLR5 separately enhanced osteoclast formation and function significantly, resulting in substantial bone loss after 5-6 days, and these effects were totally absent in the knockout mice. These results showed that activation of TLR2 or TLR5 specifically enhanced osteoclastogenesis and bone resorption. TLR2 and TLR5 stimulation also triggered massive
inflammatory infiltrate. At the gene expression level, osteoclast-related genes like those encoding cathepsin K, TRAP, c-Fos, NFATc1, CTR and Oscar were upregulated. These data demonstrate that the observed bone degradation is due to a biological response mediated by TLRs and not due to osmotic pressure change (317, 318). Interestingly, the local stimulation of TLR2 resulted in a general systemic bone loss in the rest of the skeleton, which is comparable to effect of septic arthritis, but with an important difference. In septic arthritis, the bacterium (*S. aureus*) is actually present in the blood stream and reaches every part of the skeleton, where they can activate TLR2. However, the mechanism by which TLR2 agonists cause the systemic osteoporotic effect is not known, but circulating inflammatory cytokines might be a key factor. Another possibility is the leakage of injected agonist to the blood stream. However, further investigations are needed to elucidate which humoral factor(s) is causing systemic bone loss.

*The in vivo effect by activation of TLRs is associated with increased RANKL/OPG ratio*

The same pattern of modulation by TLRs-activation of *Tnfsf11* and *Tnfrsf11b* in *vitro* was also observed in *vivo*. Stimulation of TLR2 led to upregulation of *Tnfsf11* with no effect on *Tnfrsf11b* while TLR5-induction enhanced *Tnfsf11* expression and decreased that of *Tnfrsf11b*. This consistency between the *in vivo* and *in vitro* observation, further confirm our results in contrast to those reported by Dr. Kim and colleagues that TLR2 activation inhibits *Tnfrsf11b* expression (291).

Taken all *in vitro* and *in vivo* observations together, we concluded that activation of TLRs induces osteoclast formation and bone resorption through enhanced RANKL/OPG ratio mainly in the osteoblasts. However, we cannot exclude the possible contribution of inflammatory cells in RANKL expression. We are currently trying to identify RANKL expressing cells in this model using an immunohistochemical approach.
Signaling pathway in TLR-mediated RANKL expression

Next, we sought to determine the signaling mechanism involved in upregulating RANKL expression by TLRs. To examine the role of MyD88, we used osteoblasts from MyD88 knockout animals, which revealed the indispensable action of MyD88 in this process. The MyD88 deficient osteoblasts, challenged by TLR2 or TLR5 agonists, showed no alteration in Tnfsf11 expression compared to wild type osteoblasts. 1,25(OH)2-vitamin D3, which is not known to signal through MyD88, was used as a positive control enhanced the expression of Tnfsf11 in both MyD88 null wild type osteoblasts. In addition, we studied NF-κB, the main transcription factor downstream MyD88 in TLR signaling. Activation of TLR2 and TLR5 rapidly stimulated the mRNA expression of the NF-κB subunits p50, p52, RelA (p65) and RelB in osteoblasts. It also strongly enhanced the activity of NF-κB when assessed in a reporter gene assay driving luciferase expression in osteoblasts. In addition, pharmacological inhibition of NF-κB signaling by BMS-345541 and Celastrol, which inhibit IKKs, abolished Tnfsf11 expression and the expression of the well-known NF-κB target Il6. These findings show the essential role of MyD88 and NF-κB signaling in TLR-induced Tnfsf11 expression and thereby osteoclastogenesis.

Uncoupled bone modeling by activation of TLRs in vivo

Bone formation is predominantly coupled to and often regulated by bone resorption, but during growth or physical activity (loading); it is influenced positively by systemic or mechanical factors in the absence of bone resorption. In general, inflammation near bone tissue causes bone loss, like in periodontitis and RA (8, 319). Interestingly, activation of TLR2 and TLR5 in vivo resulted in new bone formation in areas remote from the resorption loci. This phenomenon was associated with cell proliferation and upregulation of genes related to bone formation, including Col1a1, Alpl, Runx2, Bglap and Sp7. Increased expression of Runx2 protein in osteoblasts present in areas of new bone formation was confirmed using immunohistochemistry. Remarkably, the new bone formation occurs not only at the periosteal side of the skull bone, where the TLR agonists were injected, but also at the endosteal side of the bone. In contrast, TLR-activation in ex vivo organ cultured bones did not result in bone formation, but
on the contrary, in downregulation of the osteoblastic genes. These differences are most likely due to inflammation and the complex interactions and crosstalks. In conclusion, our novel observation demonstrates the uncoupled bone formation induced by inflammation via TLR2 and TLR5. However, the osteoclast-independency of this process needs to be proven and the mechanisms underlying the anabolic response remain to be studied. In preliminary analyses, we have observed increased mRNA expression of gp130 and cytokines in the IL-6 family of cytokines and of ligands and cofactors in the LRP/WNT/Frizzled signaling system and of BMP2 and TGF-β, all shown to be involved in osteoblastic bone formation.
Conclusions

Activation of TLR2 and TLR5 on osteoblasts results in bone loss associated with enhanced osteoclast formation and bone resorption, as well as with increased osteoblast differentiation and new bone formation, indicating that inflammation causes bone modeling. This thesis provides explanation to why LPS from *P. gingivalis* and flagellin from flagella-expressing bacteria can stimulate bone loss. Since TLR2 and TLR5 can be activated not only by bacterial components, but also by endogenous ligands produced in inflammatory processes, this thesis also contributes to the understanding of inflammation-induced bone loss in autoimmune diseases. Hopefully, these findings will contribute to the development of treatment strategies for inflammatory bone loss.
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Appendix (Acknowledgment)

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http://www.pgingivalis.org/ATCC33277(2).htm (Author: Tsute Chen)

**Figure 3.**
https://en.wikipedia.org/wiki/Rheumatoid_arthritis
Courtesey by A. Rydh, Radiology, Umeå
http://www.mypacs.net/cases/SEPTIC-ARTHRITIS-AND-OSTEOMYELITIS-BOTH-SIDES-OF-1ST-MTP-JOINT-803467.html (Dr. John Hunter's MSK Teaching File General Case 269)

**Figure 4.**
http://www.slideshare.net/TheSlaps/ch06-abone

**Figure 5.**
http://faculty.ivytech.edu/~shopper6/ANPweb/gallery/Week_006-4.html
Figure 6.

Figure 7A,B.

Figure 8.
Bone research society, UK. Courtesy of Kevin Mackenzie, University of Aberdeen.

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Figure 30.
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Toll-like receptors (TLRs) and inflammatory bone modeling

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