Linköping University Medical Dissertation No. 1634

Aseptic Loosening of Orthopedic Implants

Osteoclastogenesis Regulation and Potential Therapeutics

Mehdi Amirhosseini

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Linköping, Sweden 2019

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ISBN: 978-91-7685-238-5
ISSN: 0345-0082

Printed by LiU-Tryck, Linköping, Sweden, 2019

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List of papers

I. **Amirhosseini M**, Andersson G, Aspenberg P, Fahlgren A
Mechanical instability and titanium particles induce similar transcriptomic changes in a rat model for periprosthetic osteolysis and aseptic loosening
Bone Reports 2017;7:17-25

II. **Amirhosseini M**, Madsen RV, Escott KJ, Bostrom MP, Ross FP, Fahlgren A
GSK-3β inhibition suppresses instability-induced osteolysis by a dual action on osteoblast and osteoclast differentiation
J Cell Physiol. 2018;233:2398-2408

III. **Amirhosseini M**, Bernhardsson M, Lång P, Andersson G, Flygare J, Fahlgren A
Cyclin-dependent kinase 8/19 inhibition suppresses osteoclastogenesis by downregulating RANK and promotes osteoblast mineralization and cancellous bone healing
J Cell Physiol., 2019; In Press

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Populärvetenskaplig sammanfattning

Behovet av att ersätta utslitna leder med en ledprotes ökar dramatiskt i och med en åldrande befolkning. Hos majoriteten av patienterna kommer denna protes fungera hela livet ut. Dock kommer 5-7% av patienterna drabbas av att ledprotesen lossnar vilket medför ett stort lidande. I Sverige sker detta hos ca 1600 patienter årligen. Re-operationer är komplicerade och dyra. Till följd av detta, avvaktar man med att operera in ledproteser hos unga patienter vilket ger en svår livssituation för unga med artros i väntan på sin första protesoperation.

Orsaken till att proteser lossnar är komplex. Länge har man trott att proteslossning orsakas av slitagepartiklar från protesen vilket orsakar en inflammatorisk reaktion. Detta aktiverar i sin tur bennedbrytande celler (osteoklaster) samtidigt som de hämmar benuppbyggande celler (osteoblaster). På senare år har man dessutom kunnat visa att belastning som uppstår vid mekanisk instabilitet av en protes kan orsaka lika stark nedbrytning av benet som slitagepartiklar. Benvävnad behöver mekanisk belastning för normal benomsättning. När belastningen förändras så leder det till en övervikt av osteoklaster. Runt en ledprotes kan både en överbelastning såväl som en avsaknad av belastning leda till sjukliga förlopp med nedbrytning av benvävnaden.

Huvudsyftet med denna avhandling var att studera proteslossning och mekanismerna bakom mekaniskt inducerad bennedbrytning. Som kontroll jämförde vi dessa fynd med proteslossning orsakad av partiklar. Vi studerade även effekten av några möjliga läkemedelskandidater som kan hämma osteoklastaktivering och därmed har potential att stoppa eller minska bennedbrytning vid proteslossning.

För att studera proteslossning använde vi oss av en djurmodell. Ett implantat som efterliknar en ledprotes opererades in i råttans underben och utsattes för mekanisk instabilitet eller slitagepartiklar. För att studera mekanismer som orsakar proteslossning behandlade vi djuren med läkemedelskandidater som påverkar benomsättningen. Vi undersökte benvävnaden som utsatts för mekanisk instabilitet eller slitagepartiklar genom att analysera förändringar i: 1) gen- och proteinuttryck, 2) antalet benceller (osteoklaster och osteoblaster) och 3) förändring i benmassa.

Vår hypotes för den första studien var att slitagepartiklar och mekanisk instabilitet runt en protes aktiverar osteoklaster och därmed bennedbrytning via olika genregleringar. Genom att jämföra dessa två stimuli med en s.k. mikroarray, där 25 000 gener jämförs mot varandra, kunde vi visa att identiska genregleringsmönster inducerats av slitagepartiklar och mekanisk instabilitet (Studie I). Wnt-signalering är en central process vid benomsättning vid normal mekanisk aktivitet. För den andra studien använde vi vår djurmodell för att studera hur Wnt-signalering är involverad i bennedbrytningen vid proteslossning (Studie II). Genom att använda en hämmare för Wnt-signalering kunde vi bromsa mekaniskt inducerad proteslossning. Den tredje studien involverade ett helt nytt angreppssätt inom benbiologi. Detta var en studie som testade CDK-inhibitorer som tidigare hade visat ha en effekt på osteoblasterna. Dock var det fortfarande oklart om de även kunde påverka osteoklaster (Studie III). Genom omfattande försök på cell-nivå kunde vi visa att detta läkemedel inducerade både en ökad aktivitet av osteoblaster och en hämmad aktivitet av osteoklaster, vilket bekräftades genom en djurstudie som visade en ökad benmassa vid benläkning.

Betydelsen att förstå bakomliggande orsaker till proteslossning är stor då det saknas läkemedel för att stoppa proteslossning. Denna avhandling har lyckats att visa olika angreppssätt för att fördröja eller stoppa bennedbrytning vilket inkluderar nya molekylära mål för läkemedel som både påverkar osteoblaster och osteoklaster.

Popular Scientific Summary

Patients with joint disorders, either because of inflammatory diseases or overuse of joints, lose articular cartilage. This will be followed by pain and stiffness in the joints and consequently difficulty with daily activities. To restore the function of the patients, whole or parts of the joint can be replaced with a prosthesis. This procedure is called arthroplasty. During the year 2016 in Sweden more than 30,000 primary hip and knee arthroplasties were performed. While most patients are satisfied with their new artificial joint for long periods of time, in about 5-10% of patients the prosthesis will fail within 15-20 years on average. The main reason for failure of a prosthesis is aseptic loosening which is responsible for more than 50% of hip and 30% of knee prostheses reoperations. Other reasons include dislocation, periprosthetic fracture or infection. Since there are currently no drugs to prevent or stop failure of implants, the only solution for these patients is to undergo another surgical procedure, called revision arthroplasty, to renew the prosthesis. Since the aging population is growing, and thus more subjects are receiving replacement prosthesis, the number of revision arthroplasties have been increasing and are predicted to continue to do so.

A complicated combination of biological and mechanical factors causes prosthetic loosening. It is well known that wear debris particles released from the prosthetic surfaces over time will lead to a local inflammatory response. This in turn facilitates differentiation of osteoclasts, the cells that degrade bone tissue. At the same time, particles also negatively affect differentiation and function of bone forming osteoblasts. On the other hand, when bearing load during activities like walking, a prosthesis that is not fully stable in its place, will undergo micromotion. Migration of a prosthesis during the first six months after surgery is predictive for a higher risk of prosthesis loosening. This mechanical instability of prostheses can cause differentiation of osteoclast and aseptic loosening.

While the bone loss triggered by wear debris particles is studied thoroughly, little is known about the mechanisms for mechanical instability-induced prosthetic loosening. Through studies conducted in this thesis, we found that mechanical instability induces periprosthetic osteolysis, in rats, through similar cellular and molecular processes as particles are known to do. Inflammation mediated by innate immune system was found to be a shared feature between osteolysis caused by particles and by mechanical instability. Further, to explore possibilities for potential therapeutics to restrict osteolysis, we used certain compounds that target and inactivate specific mediators in signaling pathways involved in the differentiation of osteoclasts and osteoblasts. Our studies showed that inhibitors of two enzymes, GSK-3β and CDK8, can have beneficial effects on bone tissue via both suppression of osteoclast formation and promotion of osteoblast differentiation or function.

Through these studies, we gained better understanding of cellular and molecular mechanisms that orchestrate periprosthetic osteolysis and further evaluated potential therapeutic properties of compounds regulating differentiation and function of osteoclasts and osteoblasts.

Abstract

Aseptic loosening is the main cause of failure of orthopedic prostheses. With no pharmaceuticals to prevent or mitigate periprosthetic bone degradation, a surgery to replace the loose implant with a new one is the only choice to restore patients' function. Most studies on mechanisms for aseptic loosening investigate wear debris particle-induced osteolysis. However, pathological loading conditions around unstable implants can also trigger osteoclast differentiation and bone loss.

In the first study, global gene expression changes induced by mechanical instability of implants, and by titanium particles were compared in a validated rat model for aseptic loosening. Microarray analysis showed that similar signaling pathways and gene expression patterns are involved in particle- and instability-induced periprosthetic osteolysis with an early onset innate immune response as a hallmark of osteolysis induced by mechanical instability.

Further, effects of potential therapeutics on restriction of excessive osteoclast differentiation were evaluated. Wnt signaling pathway is known to regulate bone remodeling. In the second study, effects of inactivation of glycogen synthase kinase 3 beta (GSK-3β), a negative regulator of canonical Wnt signaling, on instability-induced periprosthetic osteolysis were examined using our rat model for aseptic loosening. Inhibition of GSK-3β led to a decrease in osteoclast numbers in the periprosthetic bone tissue exposed to mechanical instability while osteoblast perimeter showed an increase. This was accompanied by higher bone volume fraction (BV/TV) in animals treated with the GSK-3β inhibitor.

In the third study, potential beneficial effects of two selective inhibitors of cyclin-dependent kinase 8/19 (CDK8/19) on bone tissue were evaluated. CDK8/19 is a Mediator complex-associated transcriptional regulator involved in several signaling pathways. CDK8/19 inhibitors, mainly under investigation as treatments for tumors, are reported to enhance osteoblast differentiation and bone formation. We show in this study, for the first time, that inhibition of CDK8/19 led to marked suppression of osteoclast differentiation from bone marrow macrophages in vitro through disruption of the RANK signaling. In mouse primary osteoblasts downregulation of osteopontin mRNA, a negative regulator of mineralization, together with increased alkaline phosphatase activity and calcium deposition indicated that osteoblast mineralization was promoted by CDK8/19 inhibition. Moreover, local administration of a CDK8/19 inhibitor promoted cancellous bone regeneration in a rat model for bone healing.

These studies contribute to better understanding of mechanisms behind mechanical instability-induced periprosthetic osteolysis and propose potential therapeutics to restrict bone loss with effects on both osteoclasts and osteoblasts.

Abbreviations

ALP	Alkaline phosphatase
BMD	Bone mineral density
BMM	Bone marrow macrophage
BMP	Bone morphogenetic protein
BV/TV	Bone volume/total volume
CCL	Chemokine (C-C motif) ligand
CDK	Cyclin-dependent kinase
Col1a1	Collagen type I, alpha 1
COX-2	Cyclooxygenase 2
CTX-I	C-terminal telopeptide of type I collagen
DC-STAMP	Dendritic cell-specific transmembrane protein
Dkk	Dickkopf
ELISA	Enzyme-linked Immunosorbent assay
GSK-3β	Glycogen synthase kinase 3 beta
HMGB1	High mobility group-B1
IL	Interleukin
IPA	Ingenuity Pathway Analysis
LRP5	Low-density lipoprotein receptor-related protein 5
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage-colony stimulating factor
Micro-CT	Micro-computed tomography
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NFATc1	Nuclear factor of activated T-cells, cytoplasmic 1
NFκB	Nuclear factor κ-B
OPG	Osteoprotegerin
PMMA	Polymethyl methacrylate
Ptgs2	Prostaglandin-endoperoxide synthase 2
PTH	Parathyroid hormone
qPCR	Quantitative polymerase chain reaction
RANK	Receptor activator of NFκB
RANKL	Receptor activator of NFκB ligand
Runx2	Runt related transcription factor 2
SFRP	Secreted frizzled-related protein
THA	Total hip arthroplasty
TKA	Total knee arthroplasty
TNF	Tumor necrosis factor
TRAP	Tartrate-resistant acid phosphatase

Introduction

Total joint arthroplasty

Today, total joint arthroplasty is considered a successful surgical intervention for patients suffering from osteoarthritis, rheumatoid arthritis or other progressive joint disorders. However, around 5-10% of orthopedic implants will fail after 15-20 years and the only option to restore joint function and alleviate pain is another surgical intervention (revision) to exchange the loose implant with a new one. These operations are costly for society and strenuous for patients to undergo [1-3]. More than 30,000 primary total hip and knee arthroplasties (THA and TKA) were performed during 2016 in Sweden. The need for revision surgery after primary joint replacements has increased over time and is estimated to continue to increase in future [4]. This is mainly due to aging of population and increasing numbers of younger and physically active patients undergoing primary arthroplasties [5].

While implant failure can be a result of dislocation, mechanical loss of fixation, periprosthetic fracture, or infection, the main reason for revision arthroplasties is aseptic loosening of implants. More than half of THAs and one-third of TKAs are due to aseptic loosening [1, 3, 6, 7].

Technical factors determining the risk of aseptic loosening include surgeons' skill and experience [8], hospital volume [9, 10], materials used in bearing surfaces of implants [10-12] and implant design [13, 14]. The most important patient-related risk factor for aseptic loosening is the patients' activity level although age, gender, race, and body mass index (BMI) have also been implicated [9, 10, 15]. Initial fixation of the implants during and after the surgery is believed to be of paramount importance, as there are many reports indicating that early migration of implants can be predictive of later loosening [16-20].

In the periprosthetic tissue of aseptically loosened implants, there is an imbalance between bone degrading osteoclasts and bone forming osteoblasts resulting in excessive bone resorption and loosening of the implants. Higher numbers of osteoclasts and their precursors [21, 22] as well as elevated levels of factors promoting osteoclast differentiation like macrophage-colony stimulating factor (M-CSF) [23], receptor activator of nuclear factor κ-B (NFκB) ligand (RANKL) and RANK [24-26] are found in periprosthetic tissue in patients with loose implants. Increased expression of peptidase enzymes like cathepsin K [27] and

matrix metalloproteinases (MMPs) [28] which contribute to bone degradation by osteoclasts have been detected in periprosthetic tissue of failed prostheses. Furthermore, osteoprotegerin (OPG), produced mainly by osteoblasts, which acts as a decoy receptor for RANKL and inhibits osteoclastogenesis is found to be decreased in periprosthetic tissue of patients with aseptic loosening [29].

Etiology of periprosthetic osteolysis

The etiology of periprosthetic osteolysis has been studied extensively. There is a wide agreement that wear debris particles released from bearing surfaces of prostheses cause an inflammatory response which in the end results in osteoclast differentiation and bone loss around implants [30]. However, other causative factors like implant micromotion and high fluid pressure around implants are proposed to induce periprosthetic osteolysis [31]. Whether or not particles are the only culprit behind periprosthetic bone loss has been a matter of debate [32, 33].

Wear debris particle-induced osteolysis

Different materials are used in manufacturing of orthopedic prostheses including polyethylene, ceramic, or metals like stainless steel, titanium and cobalt-chrome-based alloys [34], as well as PMMA (polymethyl methacrylate) that can be used as bone cement for fixation of implants [35]. These can all be sources of wear debris particles that trigger an innate immune response. The most important factor determining the degree of osteolysis caused by wear debris seems to be particle load as higher concentrations of particles are associated with more osteoclastogenesis and osteolysis in tissue obtained from patients [36-39]. Size and shape of the particles have also been investigated [40, 41]. Particles isolated from periprosthetic osteolytic tissue are not uniform in size, but they are mostly in the submicron size range [42]. In vitro investigations have shown that particles from submicron size up to 10 μm can be subject to phagocytosis [43]. Smaller particles are biologically more active to induce inflammatory response and osteolysis [42, 44]. It is also suggested that

particles' contact with cell surface receptors, like CD11b/CD18, on macrophages can be sufficient to initiate the inflammatory response even without phagocytosis of particles [45].

Upon release, particles induce a foreign body granulomatous response mediated mainly by macrophages [46-48]. This involves release of several pro-inflammatory cytokines most commonly interleukin (IL)-1β, IL-6 and TNF (tumor necrosis factor). Macrophages also produce M-CSF that can directly contribute to osteoclastogenesis by promoting proliferation of monocyte/macrophage lineage cells which are osteoclast precursors (Fig. 1) [49, 50]. Both fibroblasts and osteoblasts release inflammatory cytokines like IL-6 and IL-8, as well as RANKL. Chemokines, including CCL (chemokine (C-C motif) ligand)2 and CCL3, produced by macrophages and fibroblasts in response to particle exposure also play a role by recruiting more inflammatory cells to the bone-implant interface [50]. Although T lymphocytes have been implicated in periprosthetic bone loss, they have been primarily associated with metal-on-metal implants and hypersensitivity reaction to metals [47, 51].

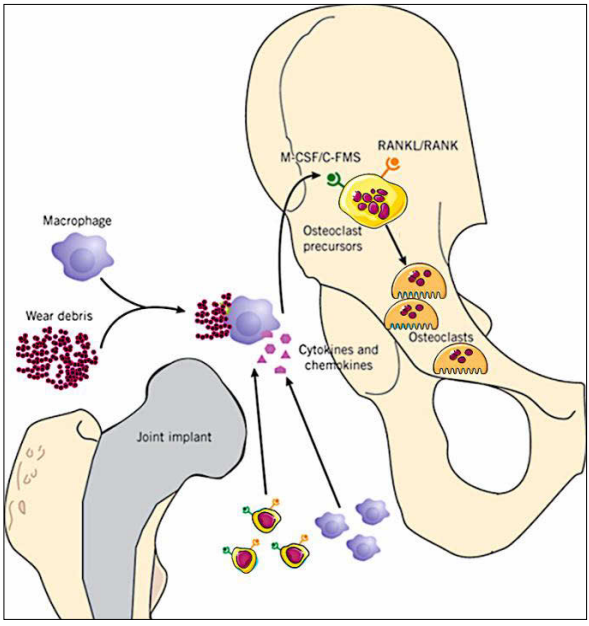

Figure 1. Wear debris particle-induced osteolysis. *Modified from ADORAble implants: Lou, K.-J. SciBX 5(25); 10.1038/scibx.2012.647.*

A series of inflammatory reactions by innate immune system to wear debris particles leads to elevated levels of RANKL, an essential factor for osteoclast differentiation, in the periprosthetic tissue [39, 40, 48, 50]. Binding of RANKL to RANK expressed on osteoclast precursors triggers cascades of signaling pathways like NFκB and MAPK (mitogen-activated protein kinase) signaling, promoting osteoclast differentiation, excessive bone resorption and consequently loosening of implants [52].

Bone loss around implants is not solely the result of unrestrained bone resorption by osteoclasts. There is evidence supporting negative effects of particles on osteoblasts' viability and function. Apoptosis in osteoblasts and decreased collagen synthesis caused by particles in in vitro investigations are reported. Furthermore, osteogenic differentiation of MSCs (mesenchymal stem cells) is reported to decrease by particles [53, 54].

In addition, osteocytes, the most abundant cell type in bone tissue, are also implicated in the response to particles. Osteocytic cell lines after exposure to particles showed increased RANKL expression [55]. In periprosthetic bone tissue of loose implants higher numbers of osteocytes with increased canalicular projections have been observed [22]. More importantly, increased osteocyte lacunar area induced by particles have been reported in osteolytic bone obtained from patients, which suggests osteocytes may directly take part in resorbing periprosthetic bone [56].

Mechanical instability-induced osteolysis

Cells in bone tissue, like most other tissues, are mechanoresponsive. Mechanical forces play a key role in modulation of bone remodeling [57]. Insertion of an implant introduces a new mechanical situation to the bone tissue surrounding it. High mechanical stress at the bone-implant interface has long been associated with loosening [58]. Mechanical instability of implants can cause micromotion [59]. Micromotion of the implant can disrupt the osseointegration process as shown by less bone ingrowth into implants in animals [60, 61]. Apart from that, clinical evidence demonstrate that implant instability is responsible for resorption of the bone surrounding implants [62]. Ex vivo application of micromotion and compression on human bone, with no particles involved, was shown to increase RANKL/OPG mRNA ratio substantially [63] indicating mechanical factors to be capable of inducing osteoclast differentiation before introduction of wear debris particles in the tissue.

Micromotion may lead to gaps at the bone-implant interface [31]. In the gaps between the implant stem and bone, a soft fibrous tissue forms that when is subjected to load (e.g. during daily walking), causes a pressurized fluid flow onto the adjacent bone. Increased prosthetic joint fluid pressure has been linked to osteolysis in patients (Fig. 2) [64]. Fluid pressure fluctuations in osteolytic lesions in patients with aseptic loosening have been recorded [65, 66]. Furthermore, patients with loose hip implants have higher intracapsular pressure compared to hips with stable implants [67]. Computational modeling experiments have suggested the fluid flow in the gaps between bone and the cement to be of supraphysiologic magnitude and able to trigger bone degradation in the absence of particles [68]. Except the proposed direct role in bone degradation, periprosthetic fluid flow is also suggested to facilitate distribution of wear particles and soluble factors promoting osteoclast differentiation at the bone-implant interface [66, 69].

The fact that early implant migration, before release of wear debris from implant surfaces, predicts later implant failure [16, 17], strengthened the idea that factors other than wear particles could be the trigger for loosening [70]. Rodent models for mechanically induced implant loosening, proposed that load-induced fluid flow and fluctuating fluid pressure at the periprosthetic bone can cause osteolysis. By mechanically loading the soft tissue between bone and implant in rabbits and rats, fluid flow created at the bone-implant interface resulted in formation of granulomatous tissue with macrophages, and bone resorption [71-75].

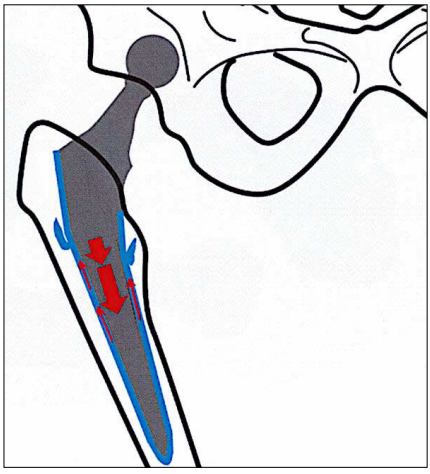

Figure 2. Schematic depiction of implant micromotion causing fluid flow under pressure in the gaps between the implant and the bone.

Aseptic loosening appears to be a multifactorial condition with complex biology. Wear debris particles and mechanical factors most probably work in concert and cause periprosthetic bone loss. It is not yet determined what initiates the process leading to bone resorption around implants. It can be speculated that micromotion, and early implant migration, are the first events that can directly induce bone degradation. Micromotion can also accelerate release of wear debris particles from prosthetic surfaces. While particle-induced osteolysis is studied extensively, signaling cascades downstream of mechanical factors causing osteolysis have been little explored.

Potential pharmacological interventions

There are currently no pharmacological interventions able to stop ongoing prosthetic loosening in patients. Efforts to develop pharmaceuticals to prevent or mitigate osteolysis around implants can be categorized into three major approaches: 1) to inhibit the inflammatory response induced by particles; 2) to inhibit osteoclast differentiation or function in the periprosthetic tissue; 3) to promote bone formation around implants.

Anti-inflammatory compounds that have been under investigation include but are not limited to inhibitors for TNF, COX-2 (Cyclooxygenase 2), and IL-1 [50, 52, 76, 77] that have shown effective in restriction of particle-induced osteolysis in mouse models. Other strategies include inhibiting recruitment of macrophages by blocking MCP-1 (monocyte chemoattractant protein 1) (CCL2) [78], or using IL-4 to shift M0 macrophage polarization towards anti-inflammatory M2 instead of pro-inflammatory M1 macrophages [79]. Glucocorticoids used in animal models suppressed osteolysis successfully [80, 81]; however, they can have serious adverse effects on endocrine system if used broadly. In general, the main drawback with anti-inflammatory treatments is possible immunosuppression. Furthermore, by the time periprosthetic bone loss is detected in patients, it is probably too late for anti-inflammatory drugs to attenuate established osteolysis [82].

Preventing excessive osteoclast differentiation around implants is another potential solution. Targeting RANK-RANKL axis using RANKL inhibitors [83] or inhibition of NFκB [84, 85], MAPK signaling [86], and NFATc1 (nuclear factor of activated T-cells, cytoplasmic 1) [87] have been effective in experimental studies. Denosumab, a human monoclonal antibody that inhibits RANKL, has shown promising results by reducing early implant migration in a

clinical trial [88]. Blunting the resorptive capacity of osteoclasts is also employed as a possible therapeutic strategy to inhibit bone resorption. Inhibitors of cathepsin K [89, 90] and MMP-9 [91] have been investigated as antiresorptive agents. Bisphosphonates, that reduce resorptive activity in mature osteoclasts, have been used in osteoporosis patients [92] to stop bone resorption. In rodent models for wear debris-induced osteolysis, they reduced bone resorption [52]. Bisphosphonates administered systemically and locally (intraoperatively), have been efficient in preventing early implant migration in clinical trials [93-95] but whether they can prevent later implant failure or not remains questionable with insufficient clinical evidence. Gene therapy strategies to restrict inflammation or osteoclastogenesis using vectors for anti-inflammatory IL-10 [96] or OPG [97] in mouse models for debris-induced osteolysis have shown promising results but viral gene therapy in humans remains a subject of controversy.

Although increase in bone resorption is the main cause for implant loosening, promoting bone formation around implants has been considered as another strategy to prevent implant failure. Parathyroid hormone (PTH), as the only approved anabolic drug for osteoporosis, showed promising results to improve implant fixation in animal studies [98, 99], but it did not efficiently reduce implant migration in clinical trials [100]. Osteogenic growth factors like BMPs (bone morphogenetic proteins) or activating Wnt signaling pathway, as promoters of bone formation, have been suggested to boost osteogenesis around implants [54, 101, 102]. The risk with systemically interfering with differentiation/function of osteoclasts and osteoblasts is disrupting the balance of bone remodeling. The aforementioned treatment strategies have shown promising results in experimental studies and some even in clinical trials; however, to date there is no approved pharmacological treatment for periprosthetic osteolysis.

Wnt signaling and bone

Since the discovery that mutations in LRP5 (low-density lipoprotein receptor-related protein 5), a Wnt coreceptor, affect bone mass, regulatory roles of Wnt signaling pathway in osteogenesis have been studied extensively [103]. Wnt signaling is primarily known to stimulate osteoblast differentiation and bone formation. When active, canonical Wnt signaling results in nuclear translocation of β-catenin. This in turn promotes transcription of

genes, in particular Runx2, that promote osteoblastogenesis (Fig. 3) [104]. However, Wnt signaling also regulates osteoclast differentiation through both canonical and noncanonical pathways [105]. Neutralizing antibodies against Wnt antagonists like Sclerostin, Dkk (Dickkopf), and SFRP (Secreted frizzled-related protein) have been under investigation as bone anabolic agents primarily to develop therapeutics for osteoporosis, fracture healing or particle-induced osteolysis [106]. No studies have evaluated how Wnt signaling modulates osteoclast and osteoblast differentiation in mechanical instability-induced osteolysis.

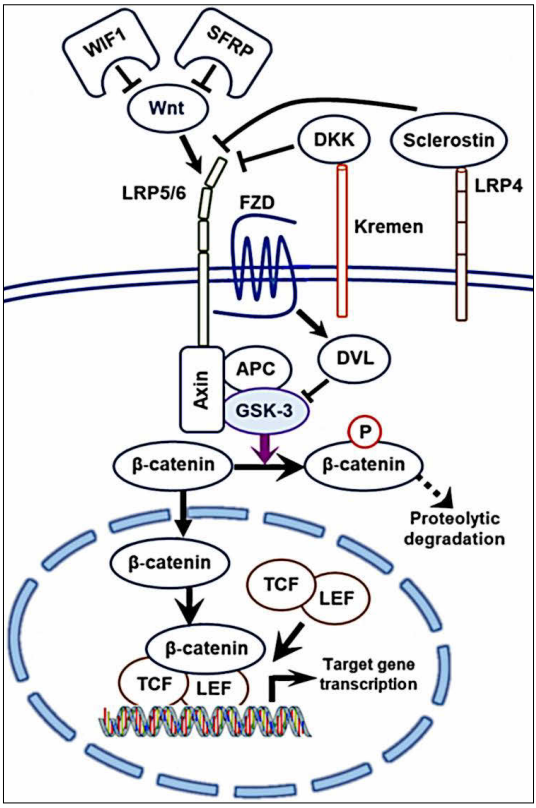

Figure 3. Canonical Wnt signaling pathway. *Modified from Baron and Kneissel, 2013* [103].

Cyclin-dependent kinase 8/19

Cyclin-dependent kinases (CDKs) control cell cycle progression. They also play roles in transcriptional regulation [107]. Inhibitors of CDKs, including CDK8/19, are under investigation as potential treatments for cancer [108]. In recent years studies reported that some CDK8/19 inhibitors can affect bone tissue [109, 110]. CDK8, and its paralog CDK19, are known as transcriptional CDKs. As part of the Mediator complex, CDK8/19 regulates RNA polymerase II-dependent transcription negatively or positively based on cell type and context. The reason for this dual action of CDK8/19 is not known yet [111-113].

The few reports on effects of CDK8/19 inhibition on bone tissue are focused on osteoblasts and bone formation. No study has yet investigated how osteoclasts are affected by CDK8/19 inhibition. Cell cycle withdrawal in osteoclast precursors is crucial for osteoclastogenesis [114, 115]. CDK8/19 plays role in the switch between proliferation and differentiation by inducing cell cycle arrest through Cip/Kip family of CDK inhibitors such as p21 and p27 [112, 116]. This suggests that CDK8/19 might be used as a target to inhibit osteoclast differentiation. However, since CDK8/19 is implicated to play role in several signaling pathways [117-119], effects of CDK8/19 are not likely to be limited to cell cycle regulation in osteoclast precursors or in osteoblasts.

Aim

General Aim

The aim of this thesis was to study the mechanisms for mechanical instability-induced periprosthetic osteolysis, and to investigate effects of potential therapeutic agents on inhibition of excessive osteoclast differentiation.

Specific Aims

- Comparing global gene expression changes during the course of periprosthetic osteolysis induced by titanium particles and mechanical instability of implants.

- Evaluating effects of GSK-3β (glycogen synthase kinase 3 beta) inhibition on osteoclast and osteoblast differentiation in an animal model for mechanical instability-induced periprosthetic osteolysis.

- Evaluating effects of CDK8/19 inhibition on differentiation and function of osteoclasts and osteoblasts in vitro, and on bone regeneration in rats.

Comments on Materials and Methods

Animal model for periprosthetic osteolysis

In this thesis (Studies I, II), a validated rat model for periprosthetic osteolysis was used (Fig. 4). This model enabled us to study osteolysis induced by mechanical instability or by wear debris particles separately.

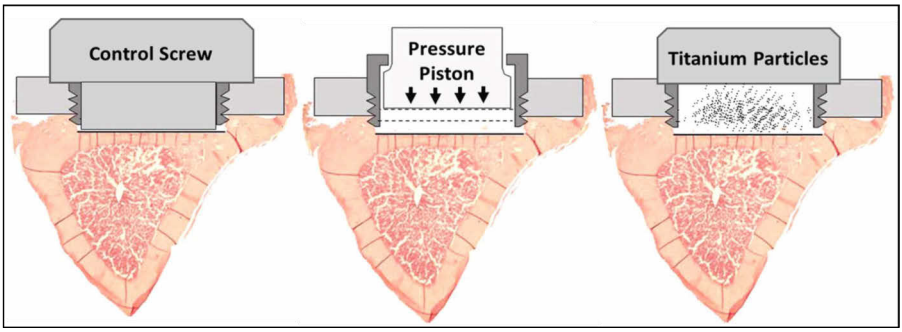

Figure 4. Transverse sections of rat proximal tibiae illustrating the validated rat model for aseptic loosening. This animal model allows simulation of mechanical instability- or particle-induced osteolysis. *From Amirhosseini et al., 2017* [120].

In this model [75, 121], on proximal tibiae of Sprague-Dawley rats, a depression is milled down onto the bone surface and a titanium plate, with a central plug (Fig. 5A), is fixed with two screws. The plate will be osseointegrated after 5 weeks. Then, in a second surgery, the central plug is removed and replaced by either a pressure piston (Fig. 5B) that recreates mechanical instability (implant micromotion), or by a hollow screw containing titanium particles (Fig. 5C) that will be released onto the underlying bone surface mimicking wear debris particles. In the control group the central plug is removed before specimen collection without application of instability or particles. Similar levels of osteoclast differentiation by induction of mechanical instability and exposure to titanium particles in this model was previously confirmed [80].

To create mechanical instability, corresponding to implant micromotion, the pressure piston is manually loaded using a dynamometer over the skin under anesthesia. This includes 20 loading cycles (pressed for 3 seconds and released for 3 seconds) with a force of 8 N at 0.17 Hz, applied within two minutes, twice a day. The piston does not reach the bone surface

during loading episodes. A 1-mm space remains between them. When loaded, the piston moves 0.5 mm down and pressurizes the interstitial fluid in the 1-mm space. This leads to a fluid flow spreading through and along the underlying bone tissue. A silicone membrane isolates the moving parts of the piston. Previous histological evaluation [80] has confirmed that no debris is released onto the bone as a result of loading episodes. While in the control animals, with the central plug, the bone tissue will be healed (Fig. 5D), induction of mechanical instability for 5 days leads to bone degradation under the implant (Fig. 5E).

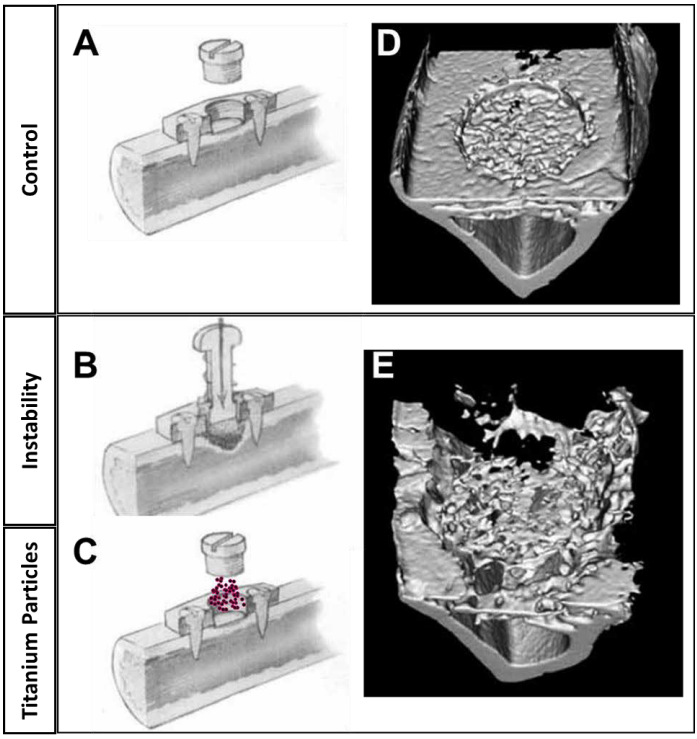

Figure 5. Schematic depiction of the animal model for aseptic loosening. *Modified from Skripitz and Aspenberg, 2000* [75]; *Fahlgren et al., 2010* [121].

Titanium particles

Wear debris particle-induced osteolysis was used as standard model to create periprosthetic osteolysis [122]. Commercially pure titanium particles were used in this animal model as wear debris particles. Titanium and its alloys have been successfully used in implant materials due to their satisfactory biocompatibility and osseointegration properties [123, 124]. In experimental studies, titanium particles are the most common in use to investigate gene expression in wear debris-induced osteolysis both in vivo and in vitro; although the concentration of applied particles and their size is not standardized in the field [125]. We used titanium particles with 90% of them < 3.6 μm in size that are biologically reactive.

Summary of the studies

Study I

Mechanical instability and titanium particles induce similar transcriptomic changes in a rat model for periprosthetic osteolysis and aseptic loosening

Aim: To compare global transcriptomic changes induced by mechanical instability and titanium particles in a rat model for aseptic loosening.

Our hypothesis was that titanium particles and mechanical instability induce periprosthetic osteolysis through different signaling pathways.

Methods:

- Sprague-Dawley rats with implants on their proximal tibiae were either administered titanium particles or subjected to implant instability.
- The bone tissue underlying the implants was harvested after 3, 48 and 120 hours from the start of the stimulation. Bone specimens were pulverized, and RNA was extracted.
- Microarray analysis was performed, and the gene expression changes induced by particles or instability compared to the unstimulated controls (fold change $\geq \pm 1.5$ and q-value ≤ 0.05) were analyzed using Ingenuity Pathway Analysis (IPA) tool.
- Microarray findings were validated by qPCR.

Main findings:

- Similar numbers of genes were differentially regulated by mechanical instability and titanium particles at each time point compared to unstimulated controls. Unexpectedly, comparing instability against particles showed no genes with a statistically significant difference at any time point.
- Functional classification of differentially regulated genes compared to unstimulated controls showed striking similarities between mechanical instability and titanium particles.

- Pathway analysis revealed no major differences between instability and particles.
- Regulated genes and pathways indicated that inflammation is a key feature of osteolysis induced by mechanical instability of implants as previously documented with wear debris particles.

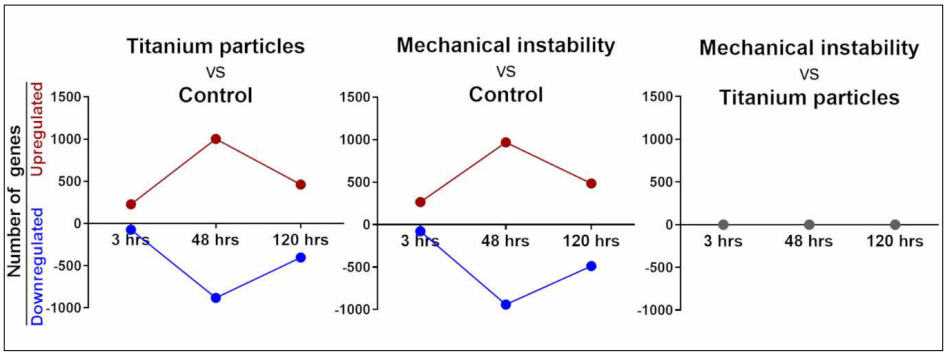

Figure 6. Similar gene expression patterns were induced by titanium particles and mechanical instability in a rat model for periprosthetic osteolysis and aseptic loosening.

Conclusions:

- Periprosthetic osteolysis caused by titanium particles and mechanical instability, in our animal model, are conducted through the same signaling pathways.
- Inflammation plays pivotal role in mechanically induced aseptic loosening.

Study II

GSK-3β inhibition suppresses instability-induced osteolysis by a dual action on osteoblast and osteoclast differentiation

Aim: To study effects of short-term inhibition of GSK-3β, a negative regulator of canonical Wnt signaling, on osteoclast and osteoblast differentiation in a rat model for instability-induced periprosthetic osteolysis.

We hypothesized that inhibition of GSK-3β will rescue mechanical instability-induced bone loss.

Methods:

- Sprague-Dawley rats were subjected to implant instability on their proximal tibiae twice daily for 3 and 5 days. Animals were orally treated (20 mg/kg bw/day) with either a selective GSK-3β inhibitor (AR28) or with vehicle. Control animals had stable implants (no loading) and received no treatments.
- The bone tissue under the implants was collected. qPCR, immunohistochemistry, histomorphometry, and micro-CT were performed to analyze markers for osteoclast and osteoblasts and changes in bone mass.

Main findings:

- Induction of mechanical instability in vehicle-treated rats, led to significant increase in number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts compared to non-loaded controls.
- The GSK-3β inhibitor decreased osteoclast numbers compared to vehicle. This correlated with a significant rise in mRNA levels of OPG resulting in decreased RANKL/OPG ratio.
- Osteoblast perimeter was increased by the GSK-3β inhibitor compared to vehicle.
- mRNA expression of β-catenin and the osteogenic markers Runx2, Osterix, collagen type I, alpha 1 (Col1a1), and ALP (alkaline phosphatase) were increased by inhibition of GSK-3β compared to vehicles.

- Mechanical instability caused downregulation of BMP-2 and Wnt16 mRNA compared to non-loaded controls. This was rescued by the GSK-3β inhibitor.
- Bone volume fraction (BV/TV) was increased following inhibition of GSK-3β compared to vehicle-treated rats.

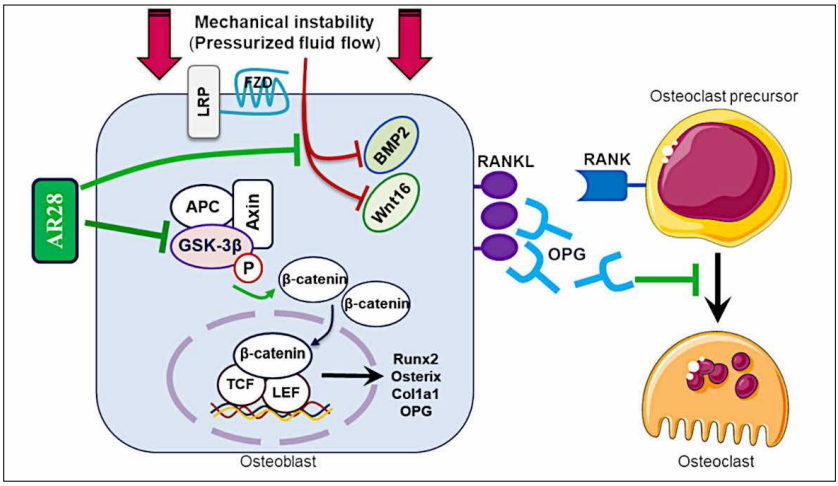

Figure 7. The GSK-3β inhibitor (AR28) promoted osteoblast differentiation and suppressed osteoclast differentiation through upregulation of OPG in a rat model for mechanical instability-induced periprosthetic osteolysis.

Conclusions:

- Inhibition of GSK-3β suppresses osteolysis induced by mechanical instability of implants.
- Inhibition of GSK-3β increases bone mass through modulating both osteoblast and osteoclast differentiation in an animal model for instability-induced aseptic loosening.

Study III

Cyclin-dependent kinase 8/19 inhibition suppresses osteoclastogenesis by downregulating RANK and promotes osteoblast mineralization and cancellous bone healing

Aim:

- To evaluate how inhibition of cyclin-dependent kinase 8/19 (CDK8/19) affects differentiation and function of osteoclasts and osteoblasts in vitro.
- To assess effects of local administration of a CDK8/19 inhibitor on bone regeneration in rats.

We hypothesized that inhibition of CDK8/19 will suppress osteoclast differentiation from mouse bone marrow macrophages in vitro and will improve bone healing in rats.

Methods:

- Mouse bone marrow macrophages (BMMs) as osteoclast precursors, and primary osteoblasts isolated from C57bl/6 mouse long bones were treated with two selective inhibitors of CDK8/19 (Senexin B and 15w).
- Osteoclast differentiation and resorption pits were assessed by TRAP and Coomassie brilliant blue staining respectively.
- TRAP activity in osteoclast culture supernatant, as well as ALP activity and calcium deposition by osteoblasts were analyzed by colorimetric assays.
- CTX-I (C-terminal telopeptide of type I collagen) levels in osteoclast culture supernatant, as well as Stat1 phosphorylation (Ser727) and total Stat1 protein levels in osteoblasts were investigated by ELISA.
- qPCR was performed to analyze gene expression changes induced by inhibition of CDK8/19 in BMMs and osteoblasts.
- NFATc1 protein levels were analyzed by Western blot.
- To assess how local treatment with a CDK8/19 inhibitor affects bone healing, Senexin B was locally administered into drill holes made on the proximal metaphysis on the tibiae of Sprague-Dawley rats, and after 14 days micro-CT was performed.

Main findings:

- Number of osteoclasts, TRAP activity and CTX-I levels in culture supernatant, as well as resorbed area on bovine bone slices were decreased by the CDK8/19 inhibitors.
- Inhibition of CDK8/19 downregulated mRNA expression of PU.1, RANK, NFκB, NFATc1, DC-STAMP (dendritic cell-specific transmembrane protein), TRAP, and cathepsin K in mouse BMMs.
- Protein levels of NFATc1 in BMMs were decreased by the CDK8/19 inhibitors.
- In primary osteoblasts ALP activity and calcium deposition was increased following inhibition of CDK8/19 accompanied by downregulation of osteopontin mRNA.
- Bone volume fraction (BV/TV) and bone mineral density (BMD) were increased by local administration of Senexin B in the drill holes made on tibiae of rats.

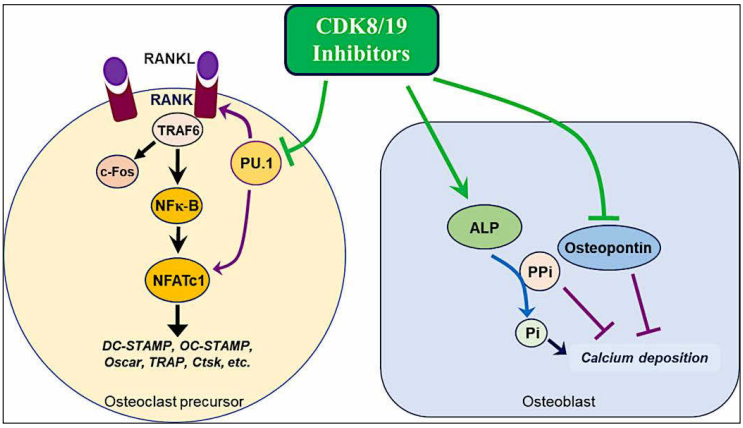

Figure 8. CDK8/19 inhibition suppressed RANKL-induced osteoclastogenesis by interrupting RANK-NFκB-NFATc1 axis and promoted osteoblast mineralization though downregulation of osteopontin and enhancing alkaline phosphatase activity.

Conclusions:

- Inhibitors of CDK8/19 suppressed RANKL-induced osteoclast differentiation by downregulating RANK signaling in osteoclast precursors.
- Through downregulating osteopontin, an inhibitor of mineralization, and elevating ALP activity, CDK8/19 inhibition enhanced mineralization by mouse primary osteoblasts.
- Local treatment with a CDK8/19 inhibitor promoted cancellous bone healing in rats.

Discussion

With no treatments available to stop osteolysis around implants, surgical intervention to revise the implant is currently the only option to alleviate pain and restore function and quality of life for patients. Revision arthroplasty is a complicated surgery with higher risk for patients and often poorer outcome compared to primary joint replacement [126]. Understanding the mechanisms for periprosthetic bone loss is necessary for developing treatments and preventive measures. Contrary to osteolysis caused by wear debris particles, mechanically induced bone loss has been studied in less detail.

We performed the first in vivo study that compares global gene expression changes, through microarray analysis, following induction of implant instability or administration of titanium particles (Study I). To our surprise, no genes were differentially regulated (fold change $\geq \pm$ 1.5 and q-value ≤ 0.05) when mechanical instability was compared to titanium particles at 3, 48, and 120 hours after stimulation. The similar gene expression patterns induced by the two stimuli were evident in the number of regulated genes by instability and particles at each time point compared to unstimulated controls. Further, when regulated genes compared to controls were classified based on their function a marked similarity was found between instability and particles. Pathway analysis using IPA also indicated similar biological processes involved in osteolysis caused by instability and particles. These were unexpected findings since these two osteolytic triggers differ by nature. We speculated that a difference in the response to mechanical instability and titanium particles can be seen at an earlier time point. We repeated the experiments and this time assessed the gene expression in bone tissue harvested 15 minutes after the stimulation. But the microarray analysis showed that no genes were regulated differentially compared to controls by either stimuli.

Confirmatory qPCR validated the same pattern as observed in microarray data in changes induced by instability and particles at 3, 48, and 120 hours in 12 key genes involved in osteoclast differentiation and function. At 15 minutes, Ptgs2 (prostaglandin-endoperoxide synthase 2) and IL-1β were upregulated significantly by mechanical instability but not by titanium particles. This implicates that a mechanical force is likely to reach and affect cells faster than wear debris particles.

Upregulation of several pro-inflammatory mediators together with pathway analysis pointed to inflammation to be a hallmark of mechanical instability-induced osteolysis. Pathways like

Acute Phase Response signaling, IL-6 signaling, Oncostatin M signaling and HMGB1 (high mobility group-B1) signaling among others were activated by both instability and particles. This suggests that implant instability in the absence of wear debris particles can elicit innate immune response and inflammation.

Although the first biologic changes after exposure to titanium particles and mechanical instability are presumably different, our data shows that only after a few hours, inflammatory responses through various mediators and pathways arise and proceed towards osteoclast differentiation in our animal model. In patients with loose implants a continuous and gradual release of particles results in sustained low level inflammation [122]. We speculate that implant micromotion and pathologic loading conditions around the implant during daily activities does the same. The inflammation mediated by various cytokines and chemokines, may be an explanation for anti-inflammatory agents targeting a single cytokine failing to rescue implant loosening.

By the time implant loosening is clinically diagnosed, the osteolysis is established and it is probably too late to target inflammation as a therapeutic approach. It seems reasonable to think that efforts should be focused on osteoclast suppression as the main target cell of the signaling pathways. Although in the course of aseptic loosening the impact of unrestrained bone resorption appears to be more prominent than blunted bone formation [88, 100], therapeutic agents that can both inhibit osteoclast differentiation/function and also promote bone formation will be preferable.

Wnt signaling modulates both osteoblast and osteoclast differentiation [104]. Hence, it has attracted interest as a target for development of bone therapeutic agents. The majority of studies on the regulatory roles of Wnt signaling in bone biology are focused on osteoporosis, fracture healing [106] or particle-induced bone loss [127, 128].

We investigated (Study II) effects of GSK-3β inactivation, using an orally available selective inhibitor (AR28), on osteoclast and osteoblast differentiation in our rat model for instability-induced periprosthetic osteolysis. GSK-3β is involved in phosphorylation and subsequent cytoplasmic degradation of β-catenin and consequently inactivation of canonical Wnt/β-catenin signaling [103]. Wnt/β-catenin signaling is involved in converting mechanical forces into biologic effects on bone tissue primarily through downregulation of sclerostin expression in osteocytes [129, 130].

The pathologic loading in our animal model, representing implant instability, stimulated significant osteoclast differentiation in the bone underneath the implant in animals treated with vehicle compared to non-loaded controls. Treatment with the GSK-3β inhibitor was able to efficiently decrease osteoclast numbers compared to vehicle. GSK-3β Inhibition also increased osteoblast perimeter accompanied by mRNA upregulation of Runx2, Osterix, Col1a1 and ALP.

The suppression of osteoclastogenesis following inactivation of GSK-3β correlated to a decreased RANKL/OPG mRNA ratio due to increased OPG expression. qPCR analysis further showed downregulation of Wnt16 and BMP-2 induced by mechanical instability in the vehicle group when compared to non-loaded controls, while by inhibiting GSK-3β, this decrease was rescued with significantly higher mRNA levels of Wnt16 and BMP-2 compared to both vehicle-treated animals and non-loaded controls.

OPG, mainly produced by osteoblasts, is reported to be a target of canonical Wnt signaling [131, 132]. Moreover, Wnt16 from osteoblasts is shown to suppress osteoclast differentiation both through direct disruption of NFκB signaling and also by upregulation of OPG [133]. Wnt16 is also shown to be upregulated in mice in response to physiological levels of mechanical loading [134]. While BMP-2 can promote β-catenin-dependent OPG expression by osteoblasts [135], it is negatively regulated by GSK-3β [136]. BMP-2, as a potent inducer of osteoblast differentiation from mesenchymal progenitor cells, is reported to cooperate with mechanical loading to enhance bone regeneration [137]. In our study, a pathologic loading regime downregulated Wnt16 and BMP-2 mRNA compared to non-loaded control animals, an effect that was abrogated by GSK-3β inhibition. The combination of decreased osteoclasts and increased osteoblasts, induced by inactivation of GSK-3β, resulted in an increase in BV/TV.

Reports on effects of GSK-3β inhibition on osteoclast differentiation in rodents are inconsistent as increased [138], decreased [139] or unaffected [140, 141] osteoclast numbers have been observed. Factors like relative potency of different inhibitors, dosage, treatment duration and intervals, route of administration, and the animal models can affect the outcome. We also tested how inhibition of GSK-3β will affect bone in intact femora of rats (without any surgery or implants) and found no significant changes in osteoclast numbers and BV/TV after 5 days. The notion that osteoclasts' role, rather than disturbed bone formation, is more prominent in the periprosthetic osteolysis, can explain suppression of osteoclastogenesis in

bone tissue subjected to implant micromotion by a GSK-3β inhibitor in our study. Other studies have shown that inhibition of GSK-3β in mice can inhibit particle-induced osteolysis [127, 142]. However, we showed for the first time that short-term inactivation of GSK-3β alleviates instability-induced bone loss through modulating both osteoblast and osteoclast differentiation.

As part of the efforts to find therapeutic agents for bone disease, cell-based screenings led to discovery of compounds showing bone anabolic effects through enhanced osteoblast differentiation [143]. Although at first the target protein of these compounds was unknown, later it was determined that they inhibit CDK8/19 [110]. Such screenings are beneficial since they can offer new, yet unexplored possibilities to develop potential therapeutic agents.

CDK8/19 acts as context-related regulator of gene expression and is also involved in the process of switch between proliferation and differentiation [111, 112] via p21 and p27 as its downstream inducers of cell cycle arrest [116]. Investigations on the effects of CDK8/19 inhibitors on bone are inadequate and limited to osteoblasts. Although reports are conflicting, there seems to be a positive effect on bone formation markers. [109, 110]. However, the mechanisms through which CDK8/19 inhibitors affect osteoblasts are yet unknown. More importantly, no studies have investigated how these compounds affect osteoclast differentiation. We investigated (Study III) how two selective inhibitors of CDK8/19 (Senexin B and 15w) affect RANKL-induced osteoclast differentiation from mouse BMMs.

Substantial decrease in osteoclast numbers, TRAP activity and CTX-I levels, a bone resorption marker, was observed following CDK8/19 inhibition. This was accompanied by mRNA downregulation of RANK, NFκB, NFATc1, DC-STAMP as well as TRAP and cathepsin K in BMMs. In mouse primary osteoblasts markers for differentiation were not affected by inhibition of CDK8/19; however, osteopontin, a negative regulator of mineralization [144] was downregulated. Additionally, ALP activity was increased. ALP contributes to mineralization through hydrolyzing inorganic pyrophosphate (PPi), a potent inhibitor of mineralization [145]. These changes resulted in enhanced mineralization by osteoblasts following inhibition of CDK8/19. Furthermore, local administration of a single dose of Senexin B led to increased BV/TV and BMD in drill holes made on rat tibiae indicating promoted bone regeneration following inhibition of CDK8/19.

Inhibitors of CDK8/19, through downregulating RANK signaling in osteoclast precursors, appear promising for further investigation to develop therapeutics in order to restrain

excessive bone resorption and possibly, if administered locally, transform the periprosthetic membrane into calcified tissue, a potential measure to rescue loose prostheses.

Concluding remarks and future

Although different in nature, wear debris particles and mechanical instability conduct periprosthetic osteolysis through similar biologic processes with inflammation as a key factor after the first few hours in our animal model. However, by the time periprosthetic bone loss is detected in patients, targeting inflammation is not likely to be the solution as the osteolysis is already established. Moreover, many inflammatory mediators appear to act in parallel towards osteoclast differentiation and targeting one does not necessarily mean the cascade is disrupted.

Blocking GSK-3β and CDK8/19 in our studies showed favorable effects on both osteoclasts and osteoblasts. Our findings offer both better understanding of mechanisms for periprosthetic osteolysis and potential therapeutic agents.

Drugs systemically administered are likely to reach the bone-implant interface with difficulty. This means high doses of drugs are needed to gain desirable effects. The challenge with use of compounds like inhibitors of GSK-3β or CDK8/19 is probable side effects with systemic treatment since they affect all cell types. Considering the risk/benefit ratio and the fact that aseptic loosening of orthopedic implants does not cause high mortality rates, systemic treatment with many of such similar compounds requires rigorous investigations and should be considered with caution.

Development of more efficient diagnostic methods, either improved imaging techniques or discovery of systemic markers, to help early diagnosis, monitor the progress of osteolysis, and evaluate response to treatments are necessary.

It is sensible to point the efforts towards preventing or postponing implant failure rather than treatment of established osteolysis around implants. Investigations aiming to find target molecules to develop therapeutics should be coupled with research on the utilization of implants as means of controlled drug delivery [146, 147] to target local periprosthetic tissue and improve osseointegration of implants. Better implant osseointegration can reduce risk of micromotion as well as wear debris production rate.

Acknowledgments

Anna Fahlgren - Göran Andersson - Per Aspenberg

Jörg Schilcher - Pernilla Eliasson - Aneta Liszka - Malin Hammerman - Franciele Dietrich - Magnus Bernhardsson - Love Tätting

Annette Molbaek - Åsa Schippert - Monika Kozak Ljunggren - Pernilla Lång - Angelina Littholt - Jenny Welander - Maria Norgård - Vesa Loitto - Anna Weinhofer

Simin Mohseni - Said Havarinasab - Parmis Blomgran - Elahe Mirrasekhian - Hammoudi Alkaissi - Lina Yakymenko Alkaissi - Ravi Kumar Dutta - Sudeep Chenna Narendra - Zaheer Ali - Anthony Mukwaya

Jonas Wetterö

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Papers

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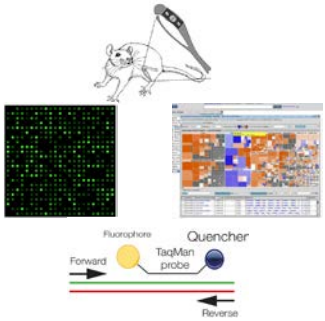
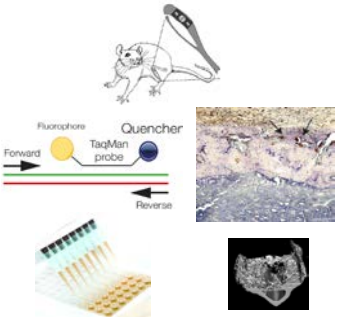
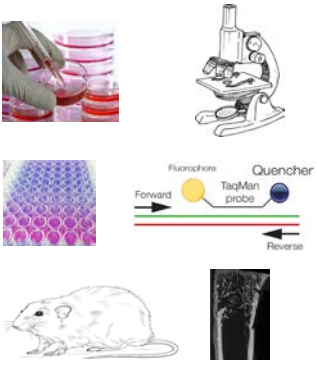
Study	Question	Answer	Methods
I	Do mechanical instability and titanium particles induce periprosthetic osteolysis through distinguished signaling pathways? What is a main feature of mechanical instability-induced periprosthetic osteolysis?	No Inflammation	
II	Does GSK-3β inhibition promote bone healing in periprosthetic osteolysis induced by mechanical instability? Does this involve modulating differentiation of both osteoblasts and osteoclasts?	Yes Yes	
III	Does CDK8 inhibition suppress osteoclast differentiation? How does CDK8 inhibition affect osteoblasts? Does local treatment with a CDK8 inhibitor improve bone regeneration in rats?	Yes Increased mineralization Yes	

FACULTY OF MEDICINE AND HEALTH SCIENCES

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