Chapter 1

General Introduction

Bone remodeling

The function of the skeleton is to give structure to the body and provide protection to vital organs such as the heart, lungs and brain. Bones also serve as a reservoir for calcium, phosphorus and magnesium and as an environment for the bone marrow where blood cells are produced. In addition, they provide attachment points for muscles and thereby facilitate movement. While bone appears to be a static tissue, it is actually constantly formed, resorbed and reformed by the different cells in the bone to adapt to changes in metabolic and mechanical requirements. This process is known as remodeling.

There are three main cell types in bone tissue involved in the remodeling of bone: the osteoclasts, osteoblasts and osteocytes (Figure 1). During remodeling, bone is resorbed by osteoclasts. Osteoclasts are large multinucleated cells that are derived from the hematopoietic cell lineage, like monocytes and macrophages. They attach to the mineralized matrix and release protons and proteolytic enzymes, which demineralize and degrade the matrix, into the space between the cell membrane and the bone. After resorption, osteoblasts are recruited to the resorbed surface to form new bone matrix. Osteoblasts originate from mesenchymal stromal cells that also give rise to myoblasts (muscle), fibroblasts (fibrous tissue), chondroblasts (cartilage)

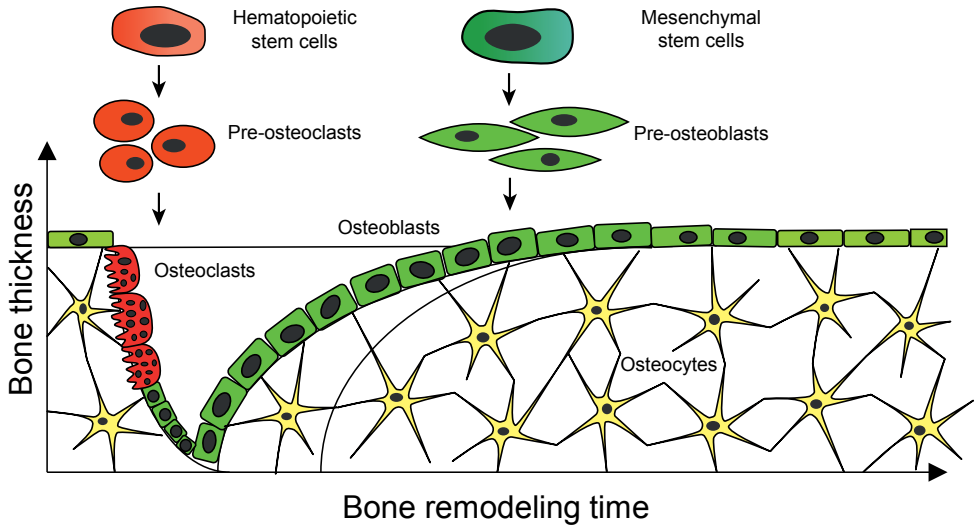

Figure 1. Bone remodeling. Osteoclasts (red) are derived from hematopoietic stem cells and resorb bone. Osteoblasts from the mesenchymal lineage (green) then fill the bone resorption pit with new bone matrix. Some osteoblasts are trapped inside the newly formed matrix and differentiate further into osteocytes (yellow).

and adipocytes (fat). During their differentiation, osteoblasts express different genes in a set order, starting with alkaline phosphatase (ALP). Later the osteoblasts synthesize proteins that form the organic matrix of bone mainly consisting of collagen type I [1]. Osteoblasts are directly involved in the mineralization of bone matrix, secreting vesicles that are rich in calcium, phosphate, alkaline phosphatase and calcium binding molecules. In these vesicles the initial production of hydroxyapatite crystals begins, and when they are released, these crystals support propagation of mineralization on the prepared matrix [2-4].

During bone formation, osteoblasts can become trapped in the matrix and differentiate further into osteocytes. Alternatively, they become quiescent bone lining cells or undergo apoptosis. Osteocytes are the most abundant cell type in bone. They are enclosed in the matrix and communicate with each other and the lining cells on the surface with long cellular processes. Because of this cellular network, osteocytes are implicated as important players in the orchestration of bone remodeling, as they are in a perfect location for sensing mechanical stress on the bone and secrete factors and transfer signals to many other bone cells.

Regulation of bone metabolism

In healthy adults, bone formation and resorption are coupled and balanced so that the total bone mass is maintained. This is tightly regulated by systemic and local factors. Systemic factors such as hormones play an important role in the regulation of bone metabolism. They meet needs of the body for calcium and phosphate by influencing both bone formation and resorption. Factors that are produced locally can affect bone cells independent from systemic hormones. Local factors control changes in metabolism to adapt to the specific requirements of the local environment. In addition, many systemic hormones have been shown to act via the production and/or activation of local factors.

Local factors

Locally produced receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF) are essential for osteoclast differentiation [5]. M-CSF promotes the survival, proliferation and differentiation of the macrophage lineage. RANKL, a membrane-bound protein produced by

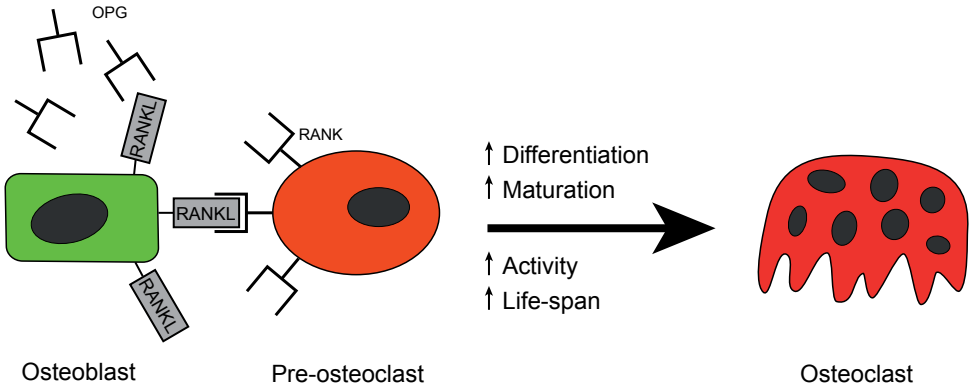

Figure 2. The RANK/RANKL/OPG system. RANKL produced by osteoblasts binds to RANK receptors on the surface of osteoclast precursors, which are then activated to differentiate and mature into osteoclasts. OPG is a protein similar to RANK, but soluble. It competes with RANK for binding to RANKL and therefore inhibits osteoclast differentiation.

osteoblasts, binds to its receptor RANK on osteoclast precursor cells and stimulates the commitment of differentiation to osteoclasts (Figure 2) [6, 7]. Osteoblasts also produce osteoprotegerin (OPG) a soluble protein that acts as a decoy receptor for RANKL and, as the name implies, protects the bone by reducing bone resorption through inhibition of osteoclast formation [8]. The ratio between RANKL and OPG determines the effect on osteoclasts. RANKL is not only essential to osteoclast differentiation and survival, it contributes to activation of mature osteoclast function as well [9]. The importance of the RANK/RANKL/OPG system was demonstrated by the effects of deletion and overexpression of these genes in animal and *in vitro* models [10]. For example, *Rank* $^{-/-}$ as well as *Rankl* $^{-/-}$ mice have a complete absence of osteoclasts with consequent shortened limbs and poorly remodeled structures blocking the marrow cavities [11, 12]. In contrast, OPG $^{-/-}$ mice showed a progressive decrease in Bone Mineral Density (BMD) and excessive osteoclast activity [13, 14]. Calcium-regulating hormones such as sex hormones, parathyroid hormone (PTH) and Vitamin D regulate expression of both RANKL and OPG and thereby control osteoclast activity [10, 15].

Different cytokines produced by cells of the immune system have an effect on both osteoblasts and osteoclasts, explaining bone effects of inflammation like the bone erosion seen in inflammatory joint disease. Activated T-cells have been shown to promote osteoclastogenesis *in vitro* by upregulation of RANKL [16, 17].

In addition to direct stimulation, T-cells produce many cytokines that stimulate (Tumor Necrosis Factor α (TNF-α), Interleukin (IL) 6 and IL-17) or inhibit (IL-4, IL-13 and IL-10) osteoclast activity and production [18].

Bone morphogenetic proteins (BMPs) were originally identified as proteins capable of induction of ectopic bone formation [19]. BMPs activate the type I and type II receptor complex, leading to initiation of signaling via phosphorylation of intracellular Smad proteins [20]. This can be inhibited by several extracellular inhibitors such as Noggin, Gremlin, Chordin and Cerberus or inhibitory Smads 6 and 7 [21]. BMP signaling has been shown to regulate the differentiation of various cells implicated in cartilage and bone formation during skeletal development and fracture repair [22-24]. Over 20 different BMPs have been identified and of these BMP2, -4, -5, -6 and -7 have been shown to induce osteoblast differentiation. In addition, BMP signaling in bone is closely linked to Wnt signaling, and many reports have shown interactions between these two pathways [25-32].

WNTs are a family of secreted proteins that regulate many developmental processes, for example body axis formation, chondrogenesis and limb development [33, 34] and have an important role in the regulation of osteoblast differentiation [35]. In the absence of Wnt activation, β-catenin is phosphorylated by glycogen synthase kinase 3 beta (GSK3β) in a complex with axin and adenomatous polyposis coli (APC) and is subsequently degraded. When WNTs bind to the Frizzled receptor and Low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor, axin is recruited to the membrane. This leads to the disruption of the destruction complex and subsequent inhibition of β-catenin phosphorylation by GSK3β. Consequently, β-catenin accumulates in the cytoplasm, translocates to the nucleus and activates the transcription of the Wnt target genes with the TCF/LEF transcription factors (Figure 3) [36, 37]. Specific deletion of β-catenin in osteocytes *in vivo* gave rise to dramatically reduced cortical bone thickness and almost absent cancellous bone, indicating the important role of Wnt/β-catenin signaling in bone formation [38]. Dickkopf-1 (DKK1) and sclerostin inhibit Wnt signaling by binding to the LRP5/6 co-receptor and thereby preventing the interaction with WNTs and the Frizzled receptor [39, 40]. Animal models have emphasized the importance of these Wnt inhibitors in regulation of bone formation. Knockout animals of both *Dkk1* and *Sost* (the gene for sclerostin) display severe gain of bone mass [41, 42], while overexpression

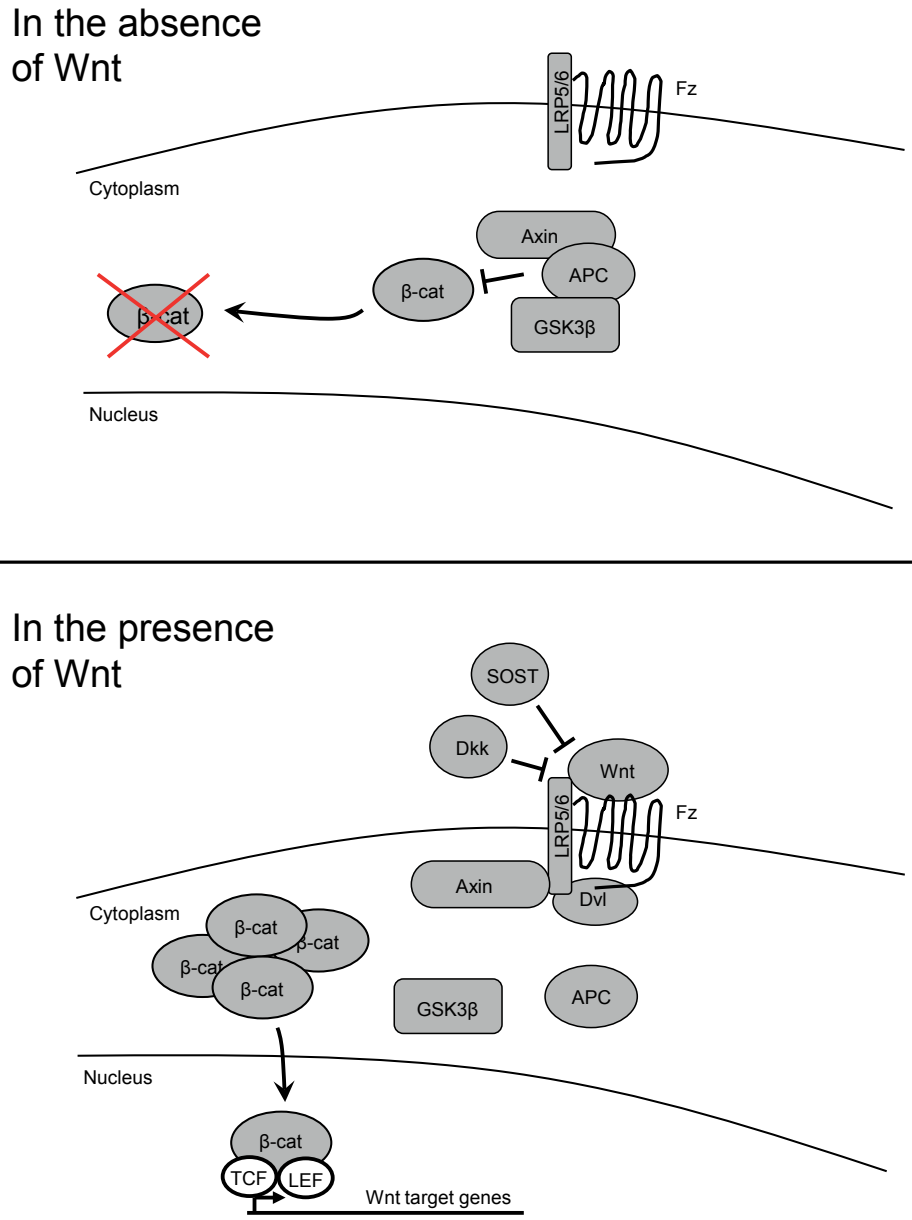

Figure 3. Wnt signaling pathway. In the absence of Wnt, GSK3β forms a complex with axin and APC to phosphorylate β-catenin, which is subsequently degraded. When Wnts are present, they bind to the Frizzled (Fz) receptor and LRP5/6 co-receptor. Axin is recruited to the membrane and the destruction complex is disrupted. β-catenin is no longer degraded and accumulates in the cytoplasm. It then translocates to the nucleus where it activates transcription of the Wnt target genes by binding to TCF/LEF transcription factors.

models showed very low bone mass [43, 44]. In humans, mutations have been found in different components of the Wnt signaling pathway. Inactivating mutations of LRP5 result in low bone mass and visual impairment (osteoporosis-pseudoglioma syndrome), while a mutation that prevents binding of the Wnt inhibitors sclerostin and DKK1 results in activation of Wnt signaling and a high bone mass phenotype [45-48]. Mutations in sclerostin or the surrounding regulatory region lead to bone overgrowth as seen in sclerosteosis and Van Buchem disease [49, 50].

Finally, bone is remodeled in response to mechanical stimuli to adapt to local loading conditions. Bone mass is lost with disuse *e.g.* in bedrest or microgravity, and gained with increasing levels of activity [51-54]. Osteocytes are thought to regulate this process by altering the production of signaling molecules after mechanical stimulation. The importance of osteocytes was demonstrated by Tatsumi *et al.* [55] who showed that loss of bone mass after hind limb unloading of mice was prevented when the majority (~80%) of osteocytes was ablated. Precisely how osteocytes sense mechanical stimuli is unknown. The current consensus is that mechanical loading induces fluid movements and shear stress in the canaliculi that surround the osteocyte processes, and this leads to changes in cytoskeleton conformation or activates stretch-activated ion channels [56].

The signaling pathways that act in osteocytes upon loading have been thoroughly investigated. Wnt/β-catenin signaling appears to play an important role as β-catenin activation is increased and *SOST* expression is decreased in bone by mechanical stimulation [57-59]. In addition, deletion of the Wnt co-receptor LRP5 reduced, while a gain-of-function mutation of LRP5 increased the response to mechanical stimulation [60, 61]. Similarly, sclerostin knockout mice are insensitive to unloading-induced bone loss [62]. PTH enhances the effect of mechanical loading, evidenced by a synergistically increased cortical bone volume in adult female mice when loading was combined with intermittent PTH(1-34) [63]. As described below, PTH may also function through modulation of sclerostin expression.

Importantly, a Wnt/LRP5 independent mechanism involving the estrogen receptor α (ERα) has also been observed [64]. The ERα was shown to be important for the response to *in vivo* loading in a study comparing expression of genes in wildtype and ERα knockout mouse bones after loading [65]. In this study, 642 genes were differentially transcribed in the tibiae of wildtype mice 3 hours after loading, while

the expression of only 26 genes was altered in the tibiae of the ERα knockout mice. The ERα was proposed to act together with the insulin growth factor-1 receptor (IGF-1R) to activate protein kinase B (PKB or AKT), which inhibits GSK3β and causes accumulation of β-catenin [66, 67].

Systemic factors

Parathyroid hormone (PTH) is produced in the parathyroid in response to low serum calcium concentrations sensed by calcium sensing receptors. It increases the reabsorption of calcium in the distal tubules in the kidney and increases production of active vitamin D leading to increased absorption of calcium in the intestine [68]. Interestingly, PTH can have both catabolic and anabolic actions on bone depending on dosage and frequency of administration. Continuous high levels of PTH in the body stimulate the PTH receptors on osteoblasts, leading to increased expression of RANKL and reduced expression of OPG [69-71]. The change in the RANKL/OPG ratio in favor of RANKL leads to increased osteoclast differentiation and bone resorption. As a result, hyperparathyroidism is a cause of secondary osteoporosis [72]. In contrast, when PTH is administered intermittently, it inhibits osteoblast apoptosis extending their matrix-synthesizing function [73]. In addition, expression of *SOST* is decreased [74-76] and this response is blunted in mice lacking the co-receptor LRP5 and in *Sost* overexpressing or deficient mice [77] suggesting an important role for sclerostin in the effect of PTH.

Loss of estrogen has long been implicated as the causative factor for the accelerated bone loss in women after menopause [78]. In women, circulating sclerostin levels are influenced by estrogen [79, 80] and estrogen has been shown to downregulate sclerostin expression in osteocyte-like cells *in vitro* [81]. The fundamental effects of estrogen on bone are to decrease bone turnover by inhibiting the initiation of remodeling and inhibit differentiation and promote apoptosis of osteoclasts. In addition, estrogen promotes osteoblast precursor commitment and differentiation and prevents osteoblast apoptosis. With estrogen withdrawal, this leads to an increase in bone remodeling and a disbalance between formation and resorption [82, 83].

Calcium is required for many functions in the body, including neuromuscular activity, membrane function, hormone secretion, enzyme activity, coagulation of

the blood and skeletal mineralization [84]. It is therefore not surprising that calcium concentrations in the blood are tightly controlled. Bone serves as a calcium store from which minerals can be drawn to maintain calcium levels when calcium intake is not sufficient to compensate for losses in urine and digestive juices [85]. Recommended daily doses differ between experts and countries, leading to wide-ranging results from 15% up to 90% of women that do not meet recommended calcium intake [86, 87]. As calcium uptake in the gut as well as food intake decrease with age, calcium supplementation in combination with vitamin D is recommended for postmenopausal women and elderly men at risk for osteoporis [88, 89]. Vitamin D is produced in the skin upon exposure to sunlight, which also accounts for shortage in the elderly and less mobile population. It increases calcium uptake in the intestine and also directly stimulates mineralization of bone matrix [90]. In addition, due to its important role in muscle function vitamin D deficiency leads to a higher risk of falling [91, 92].

A relatively new concept was presented by Gerard Karsenty and collegues in 2008 [93]. They proposed a model in which LRP5 has no direct role in bone metabolism but regulates the production of serotonin in the duodenum, and circulating serotonin inhibits bone formation. This model is actively debated as the significance of LRP5 and Wnt signaling in bone cells has been investigated in detail using (cell specific) knockout models [38, 94-100]. Cui *et al.* [101] could not replicate the results presented by Yadav *et al.* and found no relation between serotonin and bone mass. This discrepancy may be explained by differences in the mouse models that were used. At this point a definitive answer has not been found, and further research may establish whether both models can be integrated.

Osteoporosis

Disregulation of the balance between bone formation and resorption leads to an increase or reduction in bone mass, and subsequent diseases such as osteoporosis. Osteoporosis is the most common skeletal disease and is characterized by low bone mass and the loss of connectivity in the trabecular bone. This leads to decreased bone strength and increased risk of fracture, particularly in the spine, hip and wrist [102]. Osteoporosis is defined on the basis of bone mineral density (BMD) assessment of the femoral neck with dual-energy X-ray absorptiometry (DEXA). According to the

World Health Organisation criteria, osteoporosis is defined as a BMD of 2.5 standard deviations or more below the average value for young healthy women (a T-score of < -2.5 SD) [103, 104]. There are several factors that can lead to osteoporosis: low peak bone mass, accelerated bone loss, impaired bone formation during remodeling, and secondary causes like glucocorticoid use and genetic, inflammatory or nutritional disorders [105]. Peak bone mass is largely determined by genetic background, as evidenced by the large number of genetic loci associated with BMD [106, 107], but can also be influenced by lifestyle [108, 109]. Bone loss due to increased resorption is accelerated after menopause due to the loss of estrogen production and this is the main cause for development of osteoporosis in large groups of elderly females [78]. Changes in bone formation rate are inherent to ageing and may begin shortly after reaching peak bone mass [110]. The lower formation rate is probably due to changes in growth factor production and an increase in reactive oxygen species, but the precise mechanisms are unclear. In addition, inadequate calcium intake or vitamin D production decreases the bone formation rate [90].

An estimated 75 million people in Europe, the United States and Japan have osteoporosis. The disease mostly becomes apparent at a later age so due to increases in life expectancy and changing demographics this number is expected to increase worldwide, and especially in developing countries [111, 112]. In the United States, the incidence of osteoporotic fractures is higher than 1.5 million per year [113] and costs related to these fractures were estimated at 17 billion US dollars [114]. Osteoporosis mainly affects females, with only 30% of fractures occurring in males [114]. The lifetime risk for a wrist, hip, or vertebral fracture in women in the US is estimated to be 30-40% [104].

Osteoporotic fractures are most common in the hip, wrist and vertebral bones. Vertebral fractures often occur unnoticed but cause pain, deformity and long-term debility [102]. Many individuals will not regain mobility and independence after a fracture, and approximately 20% of patients will require long-term care [111, 115]. Hip fractures are known to have a high morbidity and mortality, and 5-25% of patients die within 1 year of the fracture event [102, 115, 116].

Treatment of osteoporosis

There are several established pharmacological approaches for treatment of

osteoporosis, as well as new therapies that have just been approved or are in advanced stages of clinical trials. Bisphosphonates are considered a first-line treatment for osteoporosis, and this is the most common drug class for this purpose [117]. These compounds bind strongly to hydroxyapatite in bone and have two side chains; one that participates in binding of the drug to bone and one that determines the potency and biological properties [118, 119]. During bone resorption bisphosphonates that were bound to the bone are taken up by osteoclasts and inhibit their action. Depending on the side chain the bisphosphonates have a direct toxic effect or disturb the osteoclast cytoskeleton [120, 121]. Because of their high affinity to bone, bisphosphonates are quickly bound and gradually released. They have a long half-life and can be found in plasma and urine for months or even years after the last dose [122, 123]. Most bisphosphonates are administered orally with daily or weekly dosing schedules. Oral formulations are poorly absorbed and adversely affected if taken with food or drinks. In addition they often lead to side effects like esophageal and gastric irritation [111, 117].

Bisphosphonate treatment results in decreased resorption which, through coupling mechanisms, also leads to decreased formation. The overall balance however is positive because of several reasons: 1) bone loss due to reduced bone formation is slowed in a state of decreased remodeling, 2) Slower turnover allows more time for remodeling units to finish the process of bone formation and mineralization before the site is remodeled again, 3) bone formation itself is not affected, only as a result of decreased resorption. Resorption pits that have already been formed will first be filled, leading to a transient netto increase in bone formation. 4) a decrease in resorption depth at individual remodeling sites is not matched by a decrease in local formation, and formation exceeds resorption at that location [119].

As loss of estrogen production is implicated as an important causative factor in development of osteoporosis, it seems logical to use estrogen or estrogen receptor modulators as a therapy. Indeed, hormone replacement therapy and the estrogen receptor agonist/antagonist raloxifen decreased fracture risk at both vertebral and non-vertebral sites. However, the Women's Health Initiative reported increased risk of myocardial infarction, stroke, breast cancer and deep vein thrombosis after use of these drugs. Even though a protective effect was found on endometrial cancer, these therapies are not recommended for long periods of time [124, 125].

The bioactive *N*-terminal 34-amino acid fragment of PTH (rhPTH 1-34, teriparatide) is the only bone anabolic drug currently on the market. As described above, intermittent doses of PTH stimulate osteoblast function and therefore lead to increased bone formation. The intermittent nature of administration seems to limit the effects of PTH on RANKL expression and bone resorption [126]. rhPTH affects trabecular bone more than cortical bone and therefore reduces the fracture risk of the spine much greater than that of nonvertebral bones [127]. However, it can only be used for a maximum of 2 years as after that, bone resorption catches up with formation and the drug is no longer effective. In addition, teriparatide is administered in daily subcutaneous injections and is therefore reserved for severe osteoporosis [126].

Recently, a human monoclonal antibody against RANKL (denosumab, Amgen) was approved for treatment of osteoporosis and bone destruction by bone metastases or rheumatoid arthritis [128]. Binding to RANKL, denosumab inhibits the formation, function and survival of osteoclasts and thereby inhibits bone resorption. Preclinical studies comparing denosumab to the bisphosphonate alendronate in ovariectomized mice showed that denosumab was more effective in preserving trabecular architecture and cortical thickness [129]. Clinical trials revealed marked reduction of bone turnover markers and a BMD gain at all measured sites after 1 year of treatment and a reduction in fracture risk after 3 years similar to that of the most common bisphosphonates [130]. Few specific side-effects have been reported even though RANK/RANKL also has functions in the immune system and vascular system [131]. Other than bisphosphonates, which are usually prescribed in daily or weekly tablets, denosumab is administered every 6 months by subcutaneous injections and is therefore less sensitive to adherence problems.

Neutralizing antibodies against sclerostin have been developed and are currently in phase III clinical trials (AMG 785, NCT01575834 and NCT01631214 on www.clinicaltrials.gov). Due to the restricted expression pattern of sclerostin and the good quality bone and absence of extra-skeletal complications in patients with sclerostin deficiency, sclerostin is considered a good target for bone anabolic therapy [126, 132]. Preclinical studies showed great promise increasing bone mass and strength in animal models of ovariectomized and aging animals as well as in secondary causes of osteoporosis [133-137]. In a phase I randomized double-blind

placebo-controlled study in healthy volunteers substantial and dose-dependent increases in bone formation markers and reduction of bone resorption markers were found [138]. Results from the phase II clinical trial have recently been published and reported significant increases in lumbar spine BMD at 12 months compared to placebo and, importantly, compared to the other active drugs teriparatide and alendronate [139].

Compliance is a major issue in all osteoporosis treatments. Up to 75% of patients have been reported non-compliant, and this significantly decreased therapy effectiveness [140, 141]. Determinants of compliance appear to be concerns about adverse side effects, belief in the need for medication, the relationship with the prescribing physician, administration requirements, dosing schedules and follow-up or feedback on effectiveness [142, 143].

In addition to pharmacological therapies, patients are advised to stop smoking to increase overall health, and regularly exercise to increase mechanical loading on the bones and improve muscle strength, posture and balance. This will help reduce the risk of falling and consequently fractures. Supplementation of calcium and vitamin D is also advised to achieve adequate serum levels to maintain the skeletal homeostasis [125].

Antisense oligonucleotides

Antisense oligonucleotides (AONs) are small (usually 13-25 nucleotides) RNA or DNA molecules that hybridize to a target sequence on the messenger RNA (mRNA) or pre-mRNA. AONs are well-known for their ability to induce RNAse H cleavage of the target RNA [144]. RNAse H is a ubiquitous enzyme that hydrolyzes the RNA strand of an RNA:DNA duplex. This method has been used to knock down gene expression in both experimental and clinical applications [145-148]. A 5-bp stretch of homology appears to be sufficient to induce RNAse H acitivity, and is therefore sensitive to off-target effects in longer AONs [149-151]. The only AON-based therapy currently approved is fomivirsen (Isis Pharmaceuticals), an RNAse H-inducing AON for treatment of cytomegaloviral-induced retinitis [152]. In addition, mipomersen (Genzyme) reduces APOB100 in familial hypercholesterolaemia and is under review by the European and American authorities, but there are significant side effects [153]. Custirsen (OncogeneX) inhibits clusterin, an anti-apoptotic chaperone protein

in cancer cells and is currently in phase III clinical trials [154].

While RNAse H cleavage and knockdown of genes was already a known effect of AONs in the 1980s, certain classes of AONs can have non-RNAse H mediated effects as well. For example, modulation of pre-mRNA splicing using 2'-*O*-methyl AONs was pioneered by Ryszard Kole in the 1990s [155]. His goal was to block a mutation that introduced a 'cryptic' splice site in the β-globin (HBB) and cystic fibrosis transmembrane conductance regulator (CFTR) genes and thereby restore normal splicing in patients with β-thalassemia and cystic fibrosis [155-157]. Splicing is a process in which non-coding regions (introns) are removed from the pre-mRNA to generate the messenger RNA (mRNA) with the coding regions (exons) before an RNA transcript can be translated into protein (Figure 4) [158]. Depending on for example developmental state, type of tissue or activation of cells, different (parts of) exons can be included or excluded, producing different proteins from the same gene (alternative splicing) [159, 160]. This process is controlled by sequence motifs in introns and exons that are recognized by splicing factors and can be modulated by blocking these motifs with antisense oligonucleotides (AONs).

Since this was first discovered, knowledge on the application of AONs to

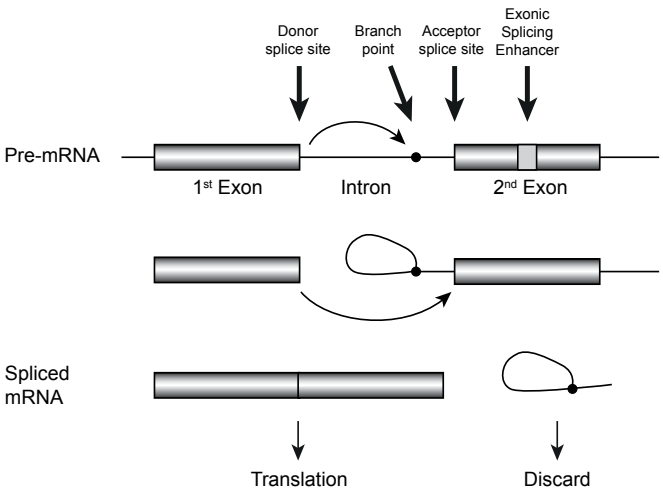

Figure 4. The process of splicing. The pre-mRNA consists of exons and introns. The end of an exon is called the donor splice site, and the beginning the acceptor splice site. A branch point sequence is located inside the intron. During splicing the branch point reacts with the donor splice site, splicing off the intron. Secondly, the donor splice site binds to the acceptor splice site and the intron is discarded. Exonic splicing enhancers (ESEs) are exon-internal sequences where proteins can bind to induce or facilitate splicing.

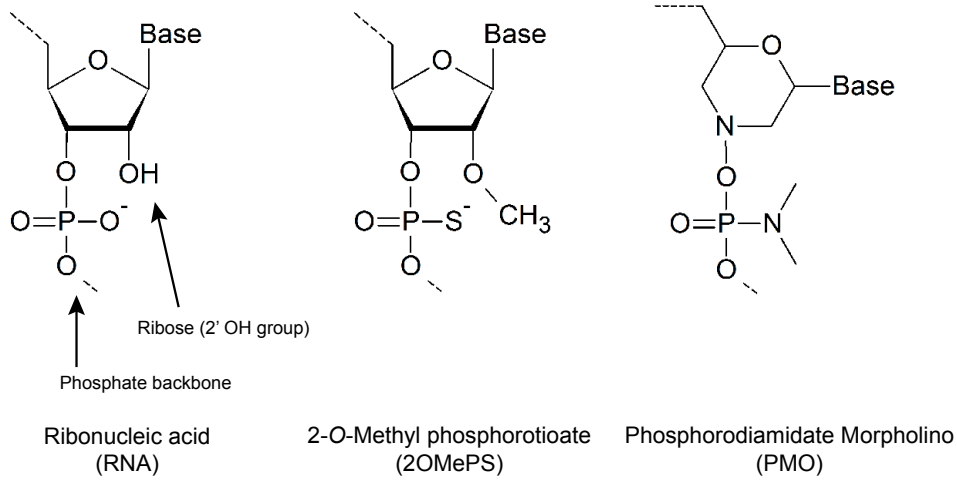

Figure 5. Frequently used AON modifications. Unmodified AONs are quickly degraded by nucleases in biological fluids. To decrease nuclease action different modifications have been developed. The 2'-*O*-Methyl phosphorothioate modification consists of a methyl group at the 2'-*O* location of the ribose, and a oxygen to sulphur substitution in the phosphate backbone. Phosphoroamidate morpholino's consist of a backbone without ribose, making them resistant to nucleases.

modulate splicing of different genes for different diseases has quickly expanded. Depending on the target, exon skipping has now also been shown to induce isoform switching, secretion of a membrane-bound protein by skipping of the membrane anchor or inactivation of a protein by removal of the active domain or disruption of the reading frame (reviewed in [161] and [148]). In addition, another class of AONs can arrest translation by steric hindrance of the ribosomal complex and consequently inhibit expression of a protein [162].

Unmodified AONs are rapidly degraded by intracellular endonucleases and exonucleases and have a short half-life [163]. Therefore, several backbone modifications have been designed to increase stability and specificity (Figure 5). The most widely used modifications for non-RNAse H mediated approaches are 1) the 2'-*O*-methyl RNA phosphorothioate (2OMePS) backbone, in which a methyl group is added to the 2'-*O* position in the ribose and one of the nonbridging oxygens is replaced by sulfur in the phosphate backbone of the oligonucleotide chain [144, 164] and 2) the phosphorodiamidate molpholino oligomers (PMOs), containing a mopholine ring and a nitrogen-based backbone [161, 165]. Phosphorothioate AONs are resistant to nucleases, easily synthesized and capable of inducing RNAse H activity for gene knockdown. The 2'-*O*-methyl modification eliminates the latter property, but further

increases stability and improves affinity for the target sequence [164]. PMOs are extremely stable, do not induce RNAse H cleavage and are thus suitable for splicing modulation. Because they are uncharged molecules, delivery of PMOs into cells in *in vitro* experiments is more difficult than 2OMePS [166]. Chimeric AONs that consist of a central region with phophorothioate backbone and a 2'-*O*-methyl backbone at the 3' and 5' ends (gapmers) can be used to induce RNAse H cleavage while retaining the favorable characteristics of the 2OMePS AONs [150, 167].

One of the most clinically advanced applications of exon skipping is practiced in treatment for boys with the severe progressive muscular disorder Duchenne muscular dystrophy (DMD). Patients are usually wheelchair-bound by the age of twelve, often require assisted ventilation later in life and generally die in their twenties [168]. The disease is caused by mutations or deletions in the DMD gene that lead to disruption of the reading frame, truncation, and loss of function of dystrophin (Figure 6) [169]. Dystrophin is required for muscle fibre stability and anchors the cytoskeleton to the extracellular matrix with two functional domains linked by a central rod domain [170, 171]. It has been demonstrated in patient cell cultures that exon skipping can be used to skip the mutated or an adjacent exon to restore the reading frame (Figure 6) [172-175]. This allows the production of dystrophins for which the central domain is shortened but both anchor domains are present, leaving it largely functional and resembling the dystrophin protein found in patients with the much milder Becker muscular dystrophy [172]. After local administration and successful dose-escalation and safety studies [176, 177], a 2OMePS AON targeted to exon 51 of DMD (GlaxoSmithKline; originally developed by the Dutch company Prosensa) is currently in Phase III clinical trials [178] (www.clinicaltrials.gov NCT01480245, NCT01462292, NCT01254019). A competing PMO AON (Sarepta (previously AVI BioPharma)) is following closely [179, 180]. Other applications of splicing-modulating AONs in early pre-clinical stages include frontotemporal dementia [181], prostate cancer [182], spinal muscular atrophy [183] and inflammatory diseases such as rheumatoid arthritis [184, 185].

Because of sequence-specificity AONs have to be designed for each new target. The first places to target for the induction of exon skipping are the donor and acceptor splice sites. However, these sites consist of consensus sequences that are shared with many different genes and therefore have a high risk of mistargeting

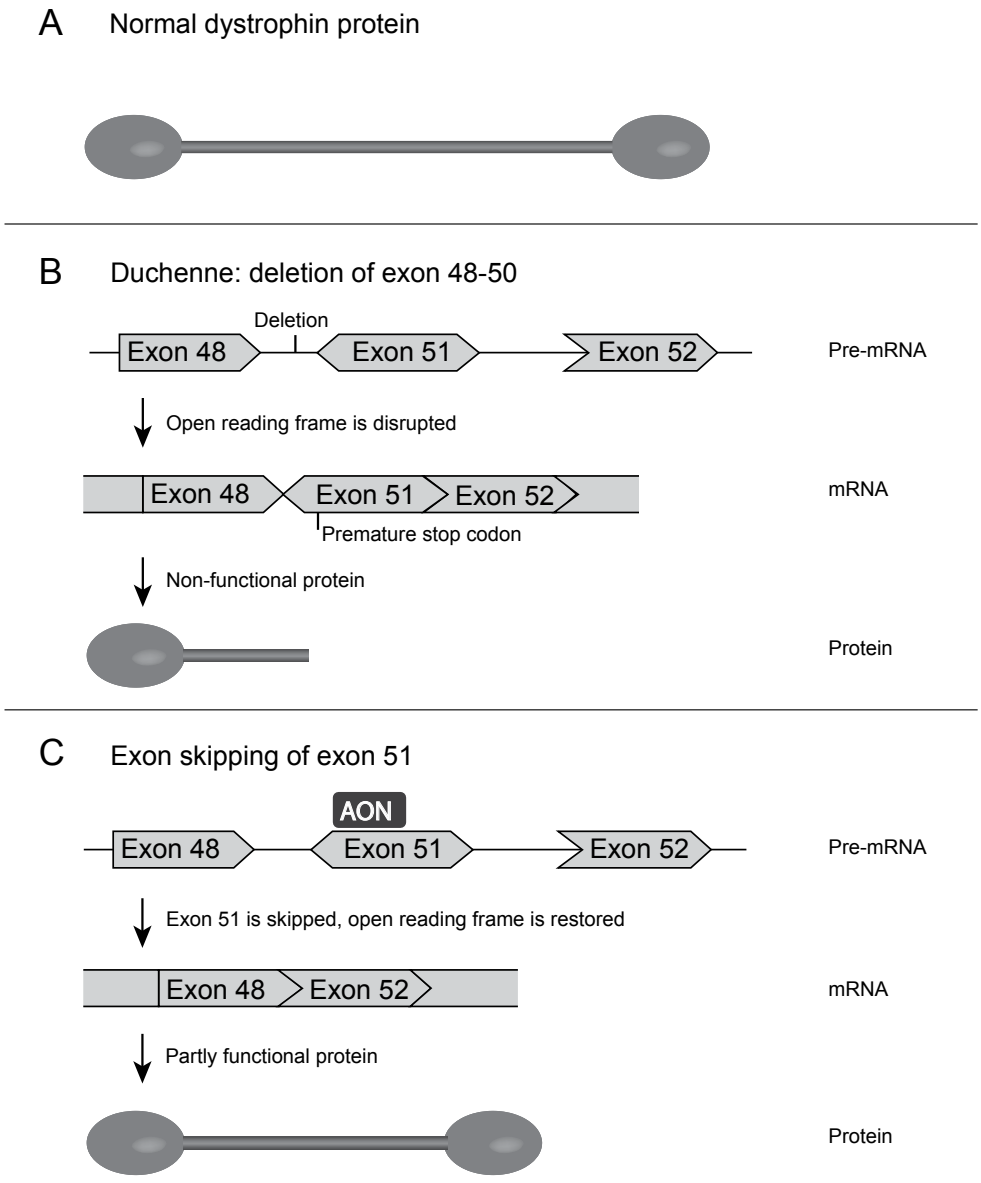

Figure 6. Exon skipping in Duchenne muscular dystrophy (DMD). Dystrophin is a large protein that contains two binding domains to link the actin in the cytoskeleton to the extracellular matrix in muscle cells and is thereby essential for muscle function (A). In DMD patients, the reading frame of the RNA is disrupted by a mutation (B). This results in a premature stop codon and loss of the linker function. In Becker dystrophy, the protein is shortened, but contains both binding domains and can partly fulfill its function. The reading frame of dystrophin in DMD patients can be restored by using AONs to induce skipping of an exon (in this case exon 51), allowing the production of Becker-like dystrophin instead of a non-functional protein (C). Figure adapted from Aartsma-Rus and van Ommen, RNA 2007 [159].

[161, 186]. In addition to the splice site sequences, many exons contain splicing regulatory sequences such as exonic splicing enhancers (ESEs), which facilitate the inclusion of exons [187]. A subfamily of splicing factors, the serine and arginine rich proteins (SR proteins), bind to these ESEs and recruit other splicing factors to the splice sites [188]. ESE motifs are loosely defined because the main task of the exon is encoding protein information, and strict motifs would interfere with this. Putative ESE sites can be predicted with software packages like RESCUE-ESE (http://genes.mit.edu/burgelab/rescue-ese/) or ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home), to help in choosing favorable sites. Based on analysis of 156 AONs that were designed for the DMD gene, several characteristics have been revealed that increase the likelihood of designing effective AONs to 65-70% [189, 190]. Around 20 nucleotides appears to be an optimal length for 2OMePS AONs, although some longer AONs can be more effective [191]. A high percentage of guanines (Gs) and cytosines (Cs) increases affinity of the AON to the target sequence, and therefore this percentage should preferable not be below 40%. However, stretches of three or more Gs or Cs or a GC percentage of >60% should be avoided as this makes self-hybridization of the AON by dimerization or folding more likely. The Tm should be above 48°C as this greatly increased efficacy in the tested DMD AONs [190]. In addition, the secondary structure of the targeted RNA sequence should be accessible for hybridization with the AON [192]. Possible secondary structures can be predicted by m-fold (http://mfold.rna.albany.edu/?q=mfold [193]). This program can also calculate the SS count for each nucleotide, indicating in how many of the predicted structures the nucleotide is single stranded. The AON should then be designed to cover partly open and partly closed structures to allow for binding to the target sequence and disruption of the secondary structure [189].

Outline of this thesis

This thesis describes different aspects of bone metabolism, mainly focused on sclerostin, Wnt signaling and bone formation. In **Chapter 2**, the current knowledge on sclerostin is reviewed and its potential as a therapeutic target for osteoporosis is highlighted. The discovery of sclerostin in patients with sclerosteosis and related mutations in Van Buchem disease have greatly improved insight in the function of sclerostin in regulation of bone metabolism. In **Chapter 3** Dkk1 levels were measured

in patients with sclerosteosis and Van Buchem disease. As this inhitibor has a similar action to sclerostin, there may be a mechanism by which Dkk1 compensates for lack of sclerostin. Further advancing knowledge is the discovery of new mutations that result in bone phenotypes. **Chapter 4** describes a novel mutation in sclerostin that results in impaired folding and secretion of the protein, and a phenotype that closely resembles sclerosteosis.

The characteristics of patients with sclerosteosis or Van Buchem disease and carriers of these diseases indicate that sclerostin would be a good target for increasing bone formation in future osteoporosis therapies. For this, sclerostin levels in osteoporosis patients will need to be persistently decreased, for example by interfering with endogenous regulatory pathways. **Chapter 5** therefore investigates the regulation of *SOST*, the gene that codes for sclerostin, and the role of GSK3β. In **Chapter 6** AONs are investigated as a method for inhibition of sclerostin expression and consequently stimulation of bone formation. In addition, bone resorption is targeted by using AONs to interfere with the function of RANK.

Further research into new therapies for osteoporosis would be aided by suitable high-throughput research methods. Since mineralization is the last and therefore a very important step in osteoblast differentiation, this is a relevant read-out. **Chapter 7** describes a new method to quantify mineralization in bone cell cultures using fluorescent probes. Finally, **Chapter 8** concludes this thesis with a general discussion.

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