Orchestration of bone remodeling

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Chapter 8

General discussion

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Osteoporosis is a growing problem in the aging society. While several therapies are available, none of these are able to persistently increase bone formation and return bone mass to a more favorable quantity that is strong enough to resist breaking. Research is therefore needed to discover new targets and develop drugs for the treatment of osteoporosis in the future.

Clinical importance

Sclerostin, a natural inhibitor of bone formation that is expressed by osteocytes, was discovered in mutation analyses of patients with sclerosteosis [1, 2]. These patients present with severe bone overgrowth and a bone mass that can be up to 11 standard deviations above normal [3]. Interestingly, the bone of these individuals is of very good quality and no affected person has been reported to have suffered a fracture [4]. Even heterozygous carriers of mutations in the gene coding for sclerostin (SOST) have consistently higher bone mass than control subjects and are relatively resistant to fracture, suggesting a gene-dose effect. The restricted expression pattern of sclerostin adds to the view of sclerostin as one of the most interesting targets for new treatment options for osteoporosis. Knowledge of the mechanism of action and regulation of sclerostin expression are essential to optimally benefit from its characteristics in future therapies. **Chapter 2** therefore gives an overview of the current knowledge on sclerostin function, expression and regulation. Even though much is known about the expression pattern and effect of sclerostin, the exact mechanism of action and pathways by which it is regulated remain to be elucidated.

A lot can be learned from mutation studies, especially in naturally occurring human diseases. In **Chapter 3** we found that Dickkopf 1 (DKK1), a protein that like sclerostin inhibits Wnt signaling through binding to Low-density lipoprotein receptor-related protein 5/6 (LRP5/6), was increased in serum samples from patients with sclerosteosis and Van Buchem disease. DKK1 may be increased in an effort to compensate for the lack of sclerostin in these patients. However, their increased bone mass indicates that the increase in DKK1 expression is insufficient to protect against excessive bone formation. While sclerostin is facilitated by LRP4 [5], DKK1 employs Kremen as a co-receptor [6, 7]. In addition, sclerostin is expressed almost exclusively in osteocytes in mineralized matrix, whereas DKK1 is expressed in many cell types

throughout the body and is implicated in tumorigenesis [8]. The proteins seem to have different roles during development. SOST knockout mice show increased bone mass without gross developmental abnormalities while mice deficient in DKK1 are embryonic lethal displaying fore- and hindlimb malformations and lacking anterior head structures [9, 10]. Therefore, even though both proteins bind to LRP5/6 they have a different mechanism of action, a different expression pattern and may act on osteoblasts at different developmental stages. This may explain the finding that DKK1 was not able to fully compensate for the lack of sclerostin.

The populations of patients with sclerosteosis and Van Buchem disease are quite isolated in South Africa and a small town in the Netherlands, respectively. After the discovery of the causative mutations for sclerosteosis and Van Buchem disease, other sclerostin mutations were found in phenotypically identical patients from Brazil, USA and Senegal [11, 12]. These mutations include several different nonsense mutations leading to a premature stop codon, and a splice site mutation interfering with correct splicing of the gene. In **Chapter 4** we described a novel missense mutation in two siblings from Turkey with classic sclerosteosis characteristics like enlarged skull and jaw, facial nerve palsy, hearing loss, headaches and highly increased bone mineral density (BMD). This mutation causes a cysteine to arginine amino acid substitution at position 167. The affected cysteine residue is the last of six cysteines forming a cystine knot and is highly conserved in multiple species. Recent data have emphasized the importance of the cystine knot motive for sclerostin function [13, 14]. The structure of sclerostin contains two fingers and a loop, emanating from the central cystine knot resembling many other cystine knot proteins [15]. This structure is highly dependent on the folding of the cystine knot. Because of the important function of the cysteine residues in protein folding, the mutation in sclerostin was predicted to disrupt the cystine knot motif and require conformational changes of the loop segment to accommodate the larger arginine side chain. These changes would lead to a global misfolding of the protein and retention in the endoplasmatic reticulum. In addition, we showed that the binding affinity of mutated sclerostin for LRP5 was decreased and its function was impaired. In the patients, this led to a phenotype comparable to that of patients with sclerosteosis. Together, these data highlight the importance of sclerostin structure and correct protein folding for its function in inhibiting Wnt signaling and bone formation.

Modulating sclerostin expression and investigating effects

As sclerostin is a negative regulator of bone formation that acts by inhibiting the Wnt signaling pathway, inhibiting the expression of this protein in patients with osteoporosis would lead to increased bone formation. By understanding the endogenous regulatory mechanisms that control *SOST* expression, we may eventually be able to specifically target and modulate these mechanisms in patients with osteoporosis to increase bone formation with minimal side-effects. In **Chapter 5** we investigated the regulation of *SOST* by modulating the activity of the Wnt signaling pathway. *SOST* expression appeared to be downregulated by increased Wnt signaling activity, and was strongly decreased by the GSK3β inhibitor GIN. However, several Wnt signaling inhibitors that work at different stages in the pathway could not abrogate the decrease in *SOST* expression. A compound that blocked the binding of β-catenin to the TCF/LEF transcription complex and thereby directly inhibited downstream target gene transcription, had no effect on the expression of *SOST* after treatment with GIN. It seems therefore that the decrease in *SOST* expression is not mediated by Wnt/β-catenin signaling, but via a different effect of GSK3β.

The importance of GSK3β in bone has been shown by inactivation and overexpression studies in mice. Global deletions of GSK3β are lethal in late embryonic or perinatal stages with various skeletal abnormalities [16-18]. Heterozygous deletions result in increased bone mass (both trabecular and cortical), and increased bone formation parameters without skeletal malformations [18, 19]. In addition to its role in Wnt/β-catenin signaling, GSK3β is also involved in insulin signaling through PKB/AKT [20]. While both pathways have been reported to induce bone formation [21, 22], GSK3β appears to be differentially regulated through the phosphorylation of different amino acids [23]. This may facilitate different downstream actions. Further research will need to identify the mechanism that GSK3β employs in the regulation of SOST.

While interfering with endogenous regulation of sclerostin expression is an interesting therapeutic option, other methods are being investigated to block the effect of the protein. New therapeutic strategies focus on increasing bone formation by inhibiting sclerostin with either antibodies or small molecules. Alternatively, interfering with transcription using antisense methods has become a common method in cell biological experiments, and efforts are being made to develop

these methods for use in the clinic. Antisense technology could therefore also be interesting in the regulation of sclerostin expression. In **Chapter 6** we investigated a novel method for the inhibition of *SOST* expression using antisense oligonucleotides (AONs). As described in detail in **Chapter 1**, AONs interfere with the splicing of a gene, leading to exclusion (skipping) of an exon from the mRNA. Depending on the exon that is skipped and its reading frame, this produces a truncated protein (out-of-frame skip) or a protein with a missing fragment (in-frame skip). As *SOST* contains only two exons, skipping of one of the exons would lead to a nonsense mRNA and obstruction of protein production. Examining 20 different AONs (both targeting *SOST* and control) did not reveal decreased *SOST* expression with any of them. In fact, several AONs increased *SOST* expression. The mechanism behind this increase is not clear, but we hypothesize that this might be due to binding of the AONs to sclerostin, triggering a compensational mechanism. Investigating the mechanism that increases *SOST* expression further may be of therapeutical interest, not to patients with osteoporosis, but instead to those with high bone mass diseases.

As bone mass is a balance between formation and resorption, therapeutic efforts could focus on either or both of these. Most available therapeutics are anti-resorptive and prevent further loss of bone mass. A relatively new anti-resorptive drug, Denosumab, targets RANKL in the RANK/RANKL/OPG pathway. This pathway is critical in the development of osteoclasts, and the anti-RANKL antibody therefore leads to a decrease in osteoclast differentiation and resorption. Denosumab showed substantial improvements in BMD and fracture risk in postmenopausal women with osteoporosis [24, 25]. In **Chapter 6** we included exon skipping of *Rank* for the decrease of resorption. Exon skipping of the membrane-binding domain of *Rank* has the added benefit that the resulting protein resembles osteoprotegerin (OPG), a decoy receptor that has an inhibitory effect on bone resorption due to reduced osteoclast differentiation. Therefore not only will *Rank* be inactivated, but a protein is formed that has an extra inhibitory effect. Unfortunately, while exon skipping was shown in several experiments, further research is required to optimize the efficiency. Only then can a biological effect be expected.

Discovering compounds that induce an increase in bone mass and strength in patients with osteoporosis would greatly improve current therapeutic options. High-throughput screening methods for relevant parameters are needed to efficiently

investigate potential candidates. For osteoblast differentiation, alkaline phosphatase (ALP) or Runx2 activity are often used as early differentiation markers. For later differentiation stages osteocalcin or sclerostin expression can be measured by RT-PCR. However, mineralization is the final and probably most fundamental step in bone formation and should therefore be the readout of choice. To provide an easy and scalable method for quantification of *in vitro* mineralization, **Chapter 7** describes a new method using fluorescent compounds Bonetag 800 and Osteosense 800. The results obtained with this method have been compared to the commonly used Alizarin Red S staining [26], and were confirmed to correlate with this method. In addition, fluorescence was specific for mineralized nodules and even seemed to be more sensitive to small changes in mineralization. Other advantages of this fluorescent method include a fast analysis, few handling or washing steps, and a readily quantifiable result.

Future perspectives of therapeutic strategies

A number of therapies have been approved for treatment of osteoporosis. Most therapies involve a decrease in bone resorption, and in this class bisphosphonates are most frequently prescribed. New therapeutic strategies that aim to increase bone formation and thereby restore bone mass, mainly focus on sclerostin as a target because of the favorable characteristics, such as its restricted expression pattern. Neutralizing antibodies against sclerostin have been shown effective in prevention of osteoporotic fractures and are in late stage clinical trials ([27] for results of the phase II trial; phase III trials NCT01575834 and NCT01631214 on www.clinicaltrials.gov), and small molecule inhibitors of sclerostin are in pre-clinical stages (www.osteogenex.com). The aim of this thesis was to increase knowledge of this interesting target and explore other therapeutic options. We therefore investigated the regulation of sclerostin expression in patients and in vitro, and the modulation of splicing using AONs. Modified AONs are a promising class of therapeutics, and many insights have been gained from the AONs used for exon skipping in patients with Duchenne muscular dystrophy (DMD). In DMD, affected muscle cells are often damaged and have leaky membranes through which AONs can easily pass [28-30]. Bone cells in patients with osteoporosis do not have this advantage. In addition, bone cells are already more difficult to reach for any compound since bone tissues

have less vasculature than muscle. Coupling AONs to specific peptides may enhance cellular uptake and targeting to certain tissues or cells [31]. Local delivery would also decrease potential systemic side effects. However, peptides may have effects of their own, leading to unexpected results or even toxicity. Indeed, clinical development of a peptide-coupled phosphorodiamidate morpholino oligomer (PMO) AON for treatment of DMD (AVI-5038) was discontinued after unexpected toxic effects were seen when high doses were administered to primates [32]. For osteoporosis, the class of bisphosphonates would be a good candidate for targeting of AONs. Bisphosphonates bind strongly to the hydroxyapatite in bone, and are therefore quickly cleared from the circulation after administration [33]. Even so, this does not automatically result in a high uptake of the AON by bone cells, as the AON would be bound to the bone matrix. By coupling bisphosphonates to AONs with a cleavable link, the AONs could be released locally by enzymatic cleavage, for example during the bone resorption process. The bisphosphonates would in this case provide a dual effect as they would also retain the antiresorptive action for which they are already used in the clinic now.

Current therapies like bisphosphonates treat osteoporosis by arresting bone resorption, but do not restore bone mass to a healthy and strong level. Drugs that stimulate bone formation would therefore be a valuable addition. This may be achieved by inhibiting sclerostin, whether by neutralizing antibodies, small molecules, modulation of gene regulation, or antisense technology. This thesis describes the development of a new quantification method for *in vitro* mineralization, which can facilitate high-throughput screening of new therapeutic compounds, the exploration of a new method of modulating expression of sclerostin, and studies which add to the knowledge of clinical significance and regulation of this protein.

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