Summary

Nederlandse samenvatting

Curriculum vitae

Summary

The skeleton has many different functions. It gives structure to the body, provides protection to vital organs and the bone marrow, serves as a reservoir for minerals and facilitates movement by providing anchor points for muscles. Bone is a living tissue that is constantly formed, resorbed and reformed by the bone cells: osteoblasts, osteoclasts and osteocytes. During the process of remodeling, osteoclasts resorb the bone matrix and new matrix is formed by osteoblasts. Some osteoblasts become trapped in the newly formed bone and differentiate into osteocytes. The osteocytes are connected to each other and to the cells on the bone surface by long cellular processes and are therefore in a perfect position to sense changes in the bone, for example loading. The osteocytes secrete factors to signal instructions to the osteoblasts and osteoclasts to react to these changes. In addition, osteoblasts and osteoclasts also produce signals and can react to some systemic hormones, which results in a complicated and not yet fully defined regulatory network that maintains the bone tissue. In **Chapter 1** some important factors in the regulation of bone metabolism are discussed.

Normally, there is a balance between resorption and formation and a constant bone mass. An imbalance can lead to an increase or decrease in bone mass, and diseases such as osteoporosis. Osteoporosis is the most common skeletal disease and is characterized by low bone mass and loss of internal bone structure. This leads to decreased bone strength and increased risk of fracture, particularly in the spine, hip and wrist. Elderly women are most at risk because bone resorption is accelerated after menopause due to the loss of estrogen production. Treatment for osteoporosis usually consists of bisphosphonates that specifically inhibit osteoclasts and therefore slow down bone resorption and disease progression. Unfortunately, bisphosphonates do not rebuild the bone to a healthy bone mass, and life-long treatment is therefore required. Other drugs are available or will probably become available in the coming years, but the ideal therapy has not yet been found and further research into the mechanisms of bone metabolism and possible therapeutic strategies is necessary.

Chapter 2 gives an overview of current knowledge on sclerostin. Sclerostin is a protein that is produced by osteocytes in mineralized bone matrix and inhibits bone formation through inhibiting Wnt signaling. It is produced by the *SOST* gene. Mutations in *SOST* or the surrounding regulatory regions and loss of

sclerostin production lead to increased bone formation. Patients with sclerosteosis or Van Buchem disease for example can present with a bone mass that is up to 11 standard deviations above normal with consequent problems such as facial paralysis and hearing loss due to facial nerve entrapment. Interestingly, the bone of these individuals is of very good quality and even heterozygous carriers of mutations in *SOST* have consistently higher bone mass than control subjects. This suggests a gene-dose effect in which a lower sclerostin production is associated with a higher bone mass. Since sclerostin is expressed specifically in osteocytes, targeting this protein in future therapies is expected to result in few side-effects. In addition, the gene-dose effect indicates that even small changes in sclerostin production could have a positive effect in patients with osteoporosis. Sclerostin is therefore one of the most interesting targets for new treatment options for osteoporosis.

Sclerostin can be measured in serum samples, and many studies have investigated serum levels in normal individuals and patients with different diseases. In patients with sclerosteosis, who have an inactivating mutation in the *SOST* gene, no sclerostin was found. Van Buchem disease is caused by a mutation in a regulatory region downstream of *SOST* and the disease is usually less severe than sclerosteosis. In accordance with this, small amounts of sclerostin were found in patients with Van Buchem disease. Dickkopf 1 (DKK1) is a protein that, like sclerostin, inhibits Wnt signaling. In **Chapter 3** we found that DKK1 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, the increase seems to be insufficient to protect against excessive bone formation.

New mutations that result in bone phenotypes can increase knowledge on the physiological actions of the involved protein. In **Chapter 4** we described a novel mutation in sclerostin after analysis of two siblings from Turkey with classic sclerosteosis characteristics. This cysteine to arginine mutation affected the last cysteine residue of the cystine-knot motif and loss of this residue probably leads to impaired folding of the protein and retention in the endoplasmatic reticulum. In addition, the mutation resulted in a significantly reduced ability to bind the Wnt co-receptor LRP5. Together, this caused a complete loss of sclerostin function and a sclerosteosis phenotype.

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* appeared to be downregulated by increased Wnt signaling activity. It was strongly decreased by GIN, which inhibits the downstream glycogen synthase kinase 3 beta (GSK3β) enzyme and thereby increases Wnt signaling activity. However, several Wnt signaling inhibitors that work at different stages in the pathway could not abrogate the decrease in *SOST* expression. In addition, directly blocking Wnt 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.

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. 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 messenger RNA (mRNA). As *SOST* contains only two exons, skipping of one of the exons would lead to a nonsense mRNA and obstruction of protein production. Unfortunately, examining 20 different AONs (both targeting *SOST* and control) did not reveal decreased *SOST* expression with one of them. A second target that was investigated in **Chapter 6** is *Rank*, which is essential in the differentiation of osteoclasts. Exon skipping of the membrane-binding domain of *Rank* has the added benefit that the resulting protein is a protein that resembles osteoprotegerin, 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. 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. 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, and were confirmed to correlate with this method.

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. In **Chapter 8** the results of this thesis are discussed in relation to current knowledge on bone metabolism and therapeutic strategies for the treatment of osteoporosis.