General Summary
The vitamin D metabolite 1,25-dihydroxyvitamin D (1,25(OH)₂D) plays a key role in the maintenance of calcium homeostasis involving actions on intestine, bone and kidney. The metabolite 1,25(OH)₂D is synthesized from the main circulating metabolite 25-hydroxyvitamin D (25(OH)D) by the renal 1α-hydroxylase enzyme, derived from the CYP27B1 gene. Low serum 25(OH)D concentrations have been associated with a lower bone mineral density, an increased bone turnover, a reduced physical performance and an increased fracture risk. To reduce the risk for falls and fractures and to optimize bone mineral density and muscle strength, an adequate serum 25(OH)D concentration is essential. However, controversy exists on the optimal serum 25(OH)D concentration in humans, which is caused by uncertainty about different thresholds that may exist for various health outcomes, such as bone mineral density and physical performance. The relationship between serum 25(OH)D and bone mineral density or physical performance may be explained by the local hydroxylation of 25(OH)D to 1,25(OH)₂D in bone and muscle, since both bone and muscle express 1α-hydroxylase. Circulating 25(OH)D may be substrate for intracellular synthesis of 1,25(OH)₂D in bone and muscle thereby affecting cell proliferation and differentiation locally after binding to the vitamin D receptor (VDR). When locally synthesized 1,25(OH)₂D positively affects osteoblast or skeletal muscle cell function, it is important to know which factors affect the local synthesis of 1,25(OH)₂D, especially for the prevention and treatment of metabolic bone diseases such as osteoporosis. However, the regulation of 1α-hydroxylase in bone and muscle cells is poorly understood. The aim of this thesis was to gain more insight into the activity of locally synthesized 1,25(OH)₂D and the factors that affect local vitamin D metabolism in bone and muscle tissue.

Both in vitro and in vivo models were used to gain more insight into the activity of locally synthesized 1,25(OH)₂D and how local vitamin D metabolism in bone and muscle is affected. With the use of a primary human osteoblast culture model, we demonstrated that incubation with 25(OH)D resulted in a reduced osteoblast proliferation and an increased expression of genes associated with osteoblast maturation and bone mineralization [chapter 2]. Osteoblasts responded to 25(OH)D by an increased expression and activity of alkaline phosphatase, an increased osteopontin expression as well as an increased osteocalcin expression and secretion. Moreover, osteoblasts expressed CYP27B1 and were able to convert 25(OH)D to 1,25(OH)₂D in a dose-dependent manner. This suggests that synthesized 1,25(OH)₂D by osteoblasts in this culture system binds to the VDR in the same cell or in neighboring cells leading to altered expression levels of genes with a VDR response element. Osteoblasts were also capable of reducing 25(OH)D and 1,25(OH)₂D concentrations by the expression of CYP24, thereby preventing an excess of vitamin D metabolites within the cell. Hydroxylation of 25(OH)D at the 24-position resulted in the formation of 24R,25-dihydroxyvitamin D (24R,25(OH)₂D) that may be involved in osteoblast differentiation as well.
Due to the autocrine and paracrine actions of 1,25(OH)\(_2\)D in osteoblasts, we hypothesized that 1\(\alpha\)-hydroxylase expression in osteoblasts is not systemically regulated by the major regulators of 1\(\alpha\)-hydroxylase in the kidney. This hypothesis was confirmed by the demonstration that CYP27B1 mRNA levels in primary human osteoblasts were not increased by parathyroid hormone (PTH) or reduced by 1,25(OH)\(_2\)D, fibroblast growth factor 23 (FGF23), calcium or phosphate [chapter 2 and 3]. Likewise, in vivo studies showed that bone CYP27B1 mRNA levels were independent of circulating 25(OH)D and 1,25(OH)\(_2\)D levels [chapter 6 and 7]. CYP24 mRNA levels in bone tissue were also not affected by circulating 25(OH)D and 1,25(OH)\(_2\)D levels [chapter 6 and 7], while osteoblasts in culture strongly increased their mRNA levels of CYP24 in the presence of 25(OH)D and 1,25(OH)\(_2\)D [chapter 2]. Moreover, CYP24 mRNA levels were not affected by PTH, FGF23, calcium and phosphate [chapter 3]. These results suggest that the regulation of 1\(\alpha\)-hydroxylase and 24-hydroxylase in bone is different from that in the kidney. The expression of 1\(\alpha\)-hydroxylase and 24-hydroxylase in bone is most likely regulated at a local level, and not by systemic factors.

Bone remodeling leads to local changes in extracellular calcium concentrations due to osteoclastic bone resorption. In chapter 3, we show that primary human osteoblasts increased their mRNA levels of CYP27B1 under high calcium conditions. Since it has been shown that high calcium concentrations in medium enhance osteoblast differentiation and matrix mineralization, we hypothesized that the higher CYP27B1 mRNA levels were due to an increase of the maturation state of osteoblasts. Indeed, mRNA levels of dentin matrix protein 1 (DMP1), an osteocyte marker, were also increased under high calcium conditions. These results suggest that CYP27B1 mRNA levels increase when osteoblasts mature. Due to the involvement of DMP1 in bone mineralization, the local synthesis of 1,25(OH)\(_2\)D may be important for matrix mineralization which is possible under high calcium conditions.

An important local factor that affects the activity of bone cells is mechanical loading. Mechanical loading causes extracellular fluid flow in the bone canalicular network resulting in osteocyte mechanosensing. Osteocytes, but in a lesser extend also osteoblasts, respond to mechanical stimuli by the production of signaling molecules. These signaling molecules affect a number of responses in bone cells which eventually may result in stimulation of osteoblast differentiation. We showed that mechanical loading of osteoblasts in the form of pulsatile fluid flow resulted in an increase of CYP27B1 mRNA levels [chapter 4]. Increased CYP27B1 mRNA levels may result in increased local 1,25(OH)\(_2\)D concentrations which in turn may lead to an enhanced osteoblast differentiation. In this way, the local synthesis of 1,25(OH)\(_2\)D may contribute to the stimulatory effect of mechanical loading on osteoblast differentiation. However, whether mechanical loading of osteoblasts, in addition to an increase of CYP27B1 mRNA levels, also results in an increased synthesis of 1,25(OH)\(_2\)D by osteoblasts, remains to be established [chapter 4].

In addition to the level of the 1\(\alpha\)-hydroxylase enzyme in osteoblasts, local con-
concentrations of 1,25(OH)$_2$D also depend on the amount of substrate. Substrate availability is probably very important since 1α-hydroxylase in osteoblasts is most likely regulated at a local level, and not systemically. In vivo, mRNA levels of CYP27B1 and CYP24 were not affected by low circulating 25(OH)D and 1,25(OH)$_2$D concentrations [chapter 6 and 7]. In case of vitamin D deficiency, low serum 25(OH)D concentrations may therefore be a limiting factor for the intracellular synthesis of 1,25(OH)$_2$D. When 25(OH)D hydroxylation in bone is an important local source for 1,25(OH)$_2$D, then a low serum 25(OH)D concentration may lead to a decreased or altered bone cell activity. However, under which circumstances a negative impact of low serum 25(OH)D levels on bone occurs, needs to be investigated.

With the use of a C2C12 mouse myoblast culture model, we demonstrated that both 25(OH)D and 1,25(OH)$_2$D reduced myoblast proliferation [chapter 5]. In C2C12 myotubes, MHC mRNA levels were increased by high concentrations of 1,25(OH)$_2$D, but 1,25(OH)$_2$D did not induce hypertrophy, change expression of myogenic regulatory factors or activate the Akt/mTOR signaling pathway. This suggests that in skeletal muscle in vivo, effects of 1,25(OH)$_2$D mainly occur in the process of satellite cell activation which is important for muscle regeneration and development, rather than in the regulation of protein turnover within myofibers. We also demonstrated that both myoblasts and myotubes expressed CYP27B1 and CYP24 mRNA, although 1α-hydroxylase activity could not be shown in myotubes. Therefore, the presence of 1α-hydroxylase activity in skeletal muscle cells needs to be addressed in another model. Due to the extremely low or absent 1α-hydroxylase activity in our culture model, the actions of 25(OH)D on C2C12 cell proliferation may be direct or indirect via conversion to 24R,25(OH)$_2$D. After all, after treatment with 1,25(OH)$_2$D or 25(OH)D myotubes showed strongly elevated CYP24 mRNA levels compared to untreated cells. Moreover, C2C12 myotubes were able to convert 25(OH)D to 24R,25(OH)$_2$D which may play a role in myoblast proliferation and differentiation.

In conclusion, bone tissue and possibly skeletal muscle tissue are capable of fine-tuning the actions of 1,25(OH)$_2$D. In osteoblasts, 25(OH)D is converted to the most active metabolite 1,25(OH)$_2$D by the enzyme 1α-hydroxylase resulting in actions on the proliferation and differentiation of cells. Intracellular concentrations of both 25(OH)D and 1,25(OH)$_2$D are reduced by 24-hydroxylase which in turn leads to a decrease of local actions of 1,25(OH)$_2$D. Both 1α-hydroxylase and 24-hydroxylase expression appear to be regulated at a local level, and not systemically, whereby bone tissue may be able to accurately regulate intracellular concentrations of the active hormone depending on their specific need at a given time. This fine-tuning mechanism of local hormone actions in peripheral target tissues appears to be a common biological principle within the endocrine system. Our results with respect to the hydroxylation of 25(OH)D to 1,25(OH)$_2$D in bone and possibly in muscle may explain the relationships of serum 25(OH)D levels with bone health outcomes and physical performance. Our results also enlarge the knowledge of the role and regula-
tion of the local vitamin D metabolism in bone and muscle which may be important for the determination of optimal serum 25(OH)D concentrations and for the prevention and treatment of osteoporosis and other metabolic bone diseases.