CHAPTER 8

General Discussion
Low serum 25(OH)D concentrations have been associated with a lower bone mineral density [1], an increased bone turnover [1], a reduced physical performance [1;2] and an increased fracture risk [3]. To reduce the risk for falls and fractures and to optimize bone mineral density and muscle strength, an adequate vitamin D status, i.e. serum 25(OH)D concentration, is essential [4]. 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 [1;5-9]. The associations of serum 25(OH)D with bone mineral density and physical performance may be explained by the local hydroxylation of 25(OH)D to 1,25(OH)\textsubscript{2}D in bone and muscle [1], since in vitro studies show expression of 1α-hydroxylase in bone and muscle [10-12]. In this way, circulating 25(OH)D may be substrate for intracellular synthesis of 1,25(OH)\textsubscript{2}D thereby affecting cell growth and differentiation in an autocrine and paracrine way [10;11;13]. When locally synthesized 1,25(OH)\textsubscript{2}D positively affects osteoblast or skeletal muscle cell function, it is important to know which factors stimulate the local synthesis of 1,25(OH)\textsubscript{2}D, 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)\textsubscript{2}D and the regulation of 1α-hydroxylation of 25(OH)D to 1,25(OH)\textsubscript{2}D in bone and muscle tissue.

LOCAL VITAMIN D METABOLISM IN BONE

Role of locally synthesized 1,25(OH)\textsubscript{2}D in bone
Bone remodeling is based on the coordinated actions of osteoblasts and osteoclasts to replace old bone with new bone [14]. Osteoblasts are derived from mesenchymal stem cells and differentiation towards mature osteoblasts is modulated by several endocrine and autocrine factors such as bone morphogenetic proteins (BMPs), growth factors, angiogenic factors, PTH and 1,25(OH)\textsubscript{2}D [14]. In chapter 2, we focused on the activity of locally synthesized 1,25(OH)\textsubscript{2}D on osteoblast differentiation. This was examined using primary human osteoblasts in culture which display characteristics of mature osteoblasts [chapter 5]. 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 [chapter 2]. Since osteoblasts express CYP27B1 and have the ability to convert 25(OH)D to 1,25(OH)\textsubscript{2}D [chapter 2;10;11], these results suggest that synthesized 1,25(OH)\textsubscript{2}D by osteoblasts in this culture system binds to the VDR in the same cell or in neighboring cells leading to altered gene expression levels [chapter
Within bone, locally synthesized 1,25(OH)$_2$D may act in an autocrine and paracrine fashion to regulate cell growth and differentiation [chapter 2;10;11].

To further elucidate the role of locally synthesized 1,25(OH)$_2$D within bone, effects of 25(OH)D on other bone cells need to be taken into account as well. Not only osteoblasts, but also osteoclasts and osteocytes express the VDR and are able to synthesize 1,25(OH)$_2$D from 25(OH)D by the presence of 1α-hydroxylase [15;16]. In primary human osteoblasts, 25(OH)D increased mRNA levels of RANKL [chapter 4], which is an essential factor for osteoclast differentiation [17;18]. In response to M-CSF and RANKL, differentiation of peripheral blood mononuclear cells into osteoclasts is induced and CYP27B1 mRNA levels in these cells increase with their differentiation [19]. Indeed, osteoclasts have the ability to synthesize more 1,25(OH)$_2$D at an increased maturation state [16]. In osteoclast cell cultures, the metabolism of 25(OH)D to 1,25(OH)$_2$D results in an increased size and TRAP activity, but a reduced resorptive activity [16;19]. This is in line with the observation that cultured osteoclasts of CYP27B1 knockout mice were smaller than those of wild type mice, but their resorptive activity was increased compared to wild-type cultures [20]. Thus, locally synthesized 1,25(OH)$_2$D may enhance osteoclast formation, but reduces the resorptive capacity of these cells [16;19;21]. In osteocytes, the role of local metabolism of 25(OH)D in osteocytes has not completely been elucidated yet, although the synthesis of 1,25(OH)$_2$D by osteocyte-like cells appears to change osteocyte gene expression consistent with an increased osteocyte maturation [15]. In addition to bone cells, the progenitors of osteoblasts, the bone marrow-derived human mesenchymal stem cells, also express 1α-hydroxylase [22]. Treatment of human mesenchymal stem cells in culture with 25(OH)D stimulates osteoblastogenesis, similar to 1,25(OH)$_2$D [23]. Thus, data from in vitro studies strongly indicate that the precursor 25(OH)D is metabolized into the active hormone 1,25(OH)$_2$D by all types of bone cells and by mesenchymal stem cells leading to a reduced bone resorption by osteoclasts, an enhanced maturation of osteoblasts and an increased matrix mineralization [chapter 2;24].

Although in vitro models contribute to a better understanding of the role of locally synthesized 1,25(OH)$_2$D within bone cells, the question remains whether the results from our in vitro study can be extrapolated to in vivo. As in humans, animal models show relationships between serum 25(OH)D levels and bone health parameters [25]. In a rat model, a positive relationship has been demonstrated between serum 25(OH)D levels and trabecular bone volume, while trabecular bone volume did not depend on serum 1,25(OH)$_2$D levels [25]. It is not expected that physiological serum concentrations of 25(OH)D result in a biological response in bone tissue, since the binding affinity for the VDR is 1000 fold lower compared to 1,25(OH)$_2$D in rodents [25]. Only supra-physiological serum concentrations of 25(OH)D (>400 nmol/l) can partially rescue the bone phenotype in CYP27B1 knockout mice, i.e. rickets [26]. Thus, the relationship between trabecular bone volume and serum 25(OH)D levels could be explained by the local synthesis of 1,25(OH)$_2$D by the 1α-hydroxylase enzyme [25].
Supportive is a normocalcemic transgenic mice study in which overexpression of the CYP27B1 gene in osteoblasts and osteocytes resulted in an increased trabecular bone volume [27]. The increased trabecular bone volume was associated with an increased bone formation rate and an unaltered osteoclast activity, suggesting that 1α-hydroxylase activity within osteoblasts and osteocytes leads to an anabolic response in bone tissue [27].

To prove the significance of CYP27B1 expression in osteoblasts in vivo, osteoblast-specific CYP27B1 knockout models may be a valuable tool. However, to our knowledge osteoblast-specific CYP27B1 knockout models have not been published yet. Regarding other extra-renal tissues, a chondrocyte-specific CYP27B1 knockout mouse model is available [28]. CYP27B1 ablation in growth plate chondrocytes affects embryonic chondrocyte development by a decreased RANKL expression and reduced osteoclastogenesis, leading to an increased width of the hypertrophic zone of the growth plate [28]. In neonatal long bones, CYP27B1 ablation in chondrocytes results in an increased bone volume and an increased expression of chondrocytic differentiation markers [28]. Additionally, the neonatal growth plate showed a reduced expression of VEGF [28]. These results strongly indicate a physiological role for the local synthesis of 1,25(OH)₂D in chondrocyte development and vascularization of the growth plate in vivo [28]. These results also support the hypothesis that locally synthesized 1,25(OH)₂D within osteoblasts does indeed have a significant function in vivo, but a CYP27B1 osteoblast-specific knockout animal model is required for the ultimate proof.

**Role of locally synthesized 24R,25(OH)₂D in bone**

Catabolism of 1,25(OH)₂D by the renal enzyme 24-hydroxylase is required for the regulation of serum 1,25(OH)₂D concentrations [29]. This was demonstrated in a CYP24-null mouse model in which fifty percent of the CYP24-null mice died in the perinatal period due to toxic levels of 1,25(OH)₂D [30], while the surviving CYP24-null mice developed impaired intramembranous bone mineralization due to elevated levels of 1,25(OH)₂D [30;31]. In addition to 1,25(OH)₂D, renal 24-hydroxylase also catabolizes 25(OH)D although the affinity for 1,25(OH)₂D is higher than that for 25(OH)D [32]. However, the enzyme 24-hydroxylase does not only regulate circulating 1,25(OH)₂D and 25(OH)D levels, but also 1,25(OH)₂D and 25(OH)D levels within the cell [33]. This was shown by osteoblasts in culture which expressed CYP24 and responded to both 25(OH)D and 1,25(OH)₂D by strongly increased CYP24 mRNA levels [chapter 2]. Although both renal and bone cells express CYP24, an important difference with renal CYP24 is that CYP24 expression in bone is independent of circulating levels of either 25(OH)D or 1,25(OH)₂D [chapter 6 and 7;34]. Other regulators of renal 24-hydroxylase, including FGF23 and PTH, do also not affect CYP24 expression in bone cells [chapter 3]. Moreover, CYP24 mRNA levels show a positive correlation with CYP27B1 mRNA levels in bone cells [35], which is in contrast to the inverse relationship between CYP24 and CYP27B1 mRNA levels in the kidney [34-36].
This implies that, similar to bone CYP27B1, the expression of CYP24 in osteoblasts is not regulated by circulating hormones, but at a local level. Thus, only local concentrations of 25(OH)D and 1,25(OH)$_2$D may be responsible for the induction of CYP24 in osteoblasts under normal physiological conditions [chapter 2;10;11]. This enables osteoblasts to reduce local 25(OH)D and 1,25(OH)$_2$D concentrations thereby preventing an excess of vitamin D metabolites within the cell.

The enzyme 24-hydroxylase is responsible for the conversion of 25(OH)D to the vitamin D metabolite 24R,25(OH)$_2$D, which has been suggested to have a physiological role in bone tissue [37]. In our culture system, primary human osteoblasts synthesized 24R,25(OH)$_2$D from 25(OH)D in a dose-dependent way [chapter 2]. In addition, incubation of primary human osteoblasts in medium supplemented with 24R,25(OH)$_2$D resulted in an increased expression of alkaline phosphatase, osteocalcin and osteopontin, suggesting that 24R,25(OH)$_2$D is able to stimulate osteoblast differentiation [chapter 2]. However, whether 24R,25(OH)$_2$D directly affects primary human osteoblast differentiation or indirectly via the metabolite 1,24R,25(OH)$_3$D which is able to increase the expression of alkaline phosphatase and osteocalcin as well [37], needs to be resolved.

Both 1,25(OH)$_2$D and 24R,25(OH)$_2$D stimulated osteoblast differentiation [chapter 2], although the potency of 24R,25(OH)$_2$D has been demonstrated to be lower than 1,25(OH)$_2$D [37]. Cultured SV-HFO osteoblasts show a lower response to 24R,25(OH)$_2$D than to 1,25(OH)$_2$D in increasing the expression of alkaline phosphatase and osteocalcin [37]. Several in vitro studies even suggest that 24R,25(OH)$_2$D has a different role than 1,25(OH)$_2$D in bone tissue [38-42]. hMSCs differentiated into osteoblasts show an enhanced mineralization in response to 24R,25(OH)$_2$D, while 1,25(OH)$_2$D was able to either inhibit or enhance mineralization depending on glucocorticoid status of the medium [42]. Actions of 24R,25(OH)$_2$D also differ from those of 1,25(OH)$_2$D regarding the increase of intracellular calcium concentrations, regulation of L-type calcium channel current, the formation of inositol phosphates as well as lipids and the production of cyclic GMP in osteoblasts. [38-41]. In vivo studies on bone fracture healing support the hypothesis that 24R,25(OH)$_2$D has a distinct physiological role from 1,25(OH)$_2$D [43]. Studies in chickens show that during fracture repair both 24-hydroxylase activity and circulating 24R,25(OH)$_2$D levels were increased [43]. Fractured tibiae in chickens fed 24R,25(OH)$_2$D in combination with 1,25(OH)$_2$D showed healing similar to that in the control group, while treatment with 1,25(OH)$_2$D alone resulted in poor healing [44]. Moreover, CYP24 ablation in mice resulted in delayed bone fracture repair which could be corrected by administration of exogenous 24R,25(OH)$_2$D [45]. Although the precise role of 24R,25(OH)$_2$D in bone tissue remains to be established, results from the above mentioned studies suggest that 24R,25(OH)$_2$D has a distinct physiological role in bone from 1,25(OH)$_2$D or it may act in concert with 1,25(OH)$_2$D to obtain an effect in bone tissue regarding processes such as osteoblast activity and fracture repair.
Regulation of CYP27B1 expression in bone

Despite of the well-described regulation of 1α-hydroxylase in the kidney, the regulation of bone 1α-hydroxylase is poorly understood. We hypothesized that 1α-hydroxylase expression found in osteoblasts is independent of regulators of renal 1α-hydroxylase, but regulated at a local level due to the autocrine and paracrine actions of synthesized 1,25(OH)_2D within bone [chapter 1 and 3]. To verify our hypothesis, we investigated whether renal regulators of 1α-hydroxylase were able to affect mRNA levels of CYP27B1 in osteoblasts [chapter 3] (Table 1). Consistent with our hypothesis, osteoblasts in culture did not respond to the major renal regulators PTH, FGF23, 1,25(OH)_2D or phosphate by altered CYP27B1 mRNA levels, as is observed in the kidney [chapter 2 and 3;46]. In bone tissue, CYP27B1 mRNA levels were also independent of serum concentrations of 1,25(OH)_2D [chapter 6 and 7]. Our results confirm the hypothesis that, although both renal and bone cells express identical 1α-hydroxylase proteins, the regulation of CYP27B1 expression in osteoblasts differs from that in renal cells [chapter 3].

CYP27B1 expression in osteoblasts is most likely regulated by local factors derived from bone cells or cells from the bone marrow [chapter 3]. In extra-renal cells such as keratinocytes, macrophages and endothelial cells, local factors including cytokines and growth factors appear to be involved in the regulation of 1α-hydroxylase [13;47]. In osteoblasts, CYP27B1 expression is stimulated by interleukin-1β (IL-1β) which is mainly produced by monocytes and macrophages, but also by osteocytes [48]. IL-1β is an important mediator of the inflammatory response and involved in a variety of processes such as cell proliferation and differentiation [48]. Reduction of CYP27B1 expression has been shown by transforming growth factor-β (TGF-β) and insulin-like growth factor-1 (IGF-1) [49]. These are growth factors produced by bone cells and play a role in osteoblast proliferation and differentiation [49].

An important local factor that affects the activity of bone cells is mechanical loading [50]. Osteocytes, but in a lesser extend also osteoblasts, respond to mechanical stimuli by the production of signaling molecules [51-53]. These signaling molecules affect a number of responses in bone cells which eventually may result in stimulation of osteoblast differentiation [51;53;54]. 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 subsequently result in increased local 1,25(OH)_2D concentrations leading to an enhanced osteoblast differentiation. In this way, the local synthesis of 1,25(OH)_2D may contribute to the stimulatory effect of mechanical loading on osteoblast differentiation. However, whether mechanical loading of osteoblasts results in an increased synthesis of 1,25(OH)_2D by osteoblasts remains to be established [chapter 4].

In addition to regulatory factors, CYP27B1 expression is dependent on the maturation state of the osteoblast [35;55]. Osteoblasts show an increased expression of CYP27B1 in late mature and early mineralizing cultures [35]. Moreover, the increased
Table 1. Regulation of 1α-hydroxylase in human mesenchymal stem cells, osteoblasts and osteocytes.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Factor</th>
<th>Conc.</th>
<th>Effect</th>
<th>RNA/protein /activity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMSCs</td>
<td>PTH1-34</td>
<td>100 nM</td>
<td>↑</td>
<td>RNA/activity</td>
<td>[118]</td>
</tr>
<tr>
<td></td>
<td>IGF-1</td>
<td>100 ng/ml</td>
<td>↑</td>
<td>protein</td>
<td></td>
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<tr>
<td>hMSCs</td>
<td>1,25(OH)₂D</td>
<td>0.01-10 nM</td>
<td>↓</td>
<td>RNA</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>25(OH)D</td>
<td>0.01-125 nM</td>
<td>↑</td>
<td>RNA</td>
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<tr>
<td>SV-HFO osteoblasts</td>
<td>25(OH)D</td>
<td>10-200 nM</td>
<td>−</td>
<td>RNA</td>
<td>[10]</td>
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<tr>
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<td>↑</td>
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<tr>
<td></td>
<td>CaCl₂</td>
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<td>−</td>
<td>RNA</td>
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<td>PTH</td>
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<td>−</td>
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<tr>
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<td></td>
<td>1000 nM</td>
<td>↓</td>
<td>RNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000 nM</td>
<td>−</td>
<td>RNA</td>
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<td></td>
<td>1000 nM</td>
<td>↓</td>
<td>RNA</td>
<td></td>
</tr>
<tr>
<td>MLO-A5</td>
<td>25(OH)D</td>
<td>100 nM</td>
<td>↓</td>
<td>RNA</td>
<td>[61]</td>
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<tr>
<td></td>
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<td>1 nM</td>
<td>↓</td>
<td>RNA</td>
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<tr>
<td>ROS 17/2.8</td>
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<td>luciferase activity</td>
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<td>IGF-1</td>
<td>1 nM</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary human osteoblasts</td>
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<td>100-400 nM</td>
<td>−</td>
<td>RNA</td>
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<td>RNA</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Primary human osteoblasts</td>
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<td>RNA</td>
<td>[chapter 3]</td>
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<td>−</td>
<td>RNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaH₂PO₄</td>
<td>0.5-3.0 mM</td>
<td>−</td>
<td>RNA</td>
<td></td>
</tr>
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</table>
expression of CYP27B1 is associated with increased expression levels of genes involved in osteoblast maturation and mineralization [35]. This suggests that the locally synthesized 1,25(OH)\textsubscript{2}D is involved in osteoblast maturation and mineralization [35]. In chapter 3, we show that primary human osteoblasts increased their mRNA levels of CYP27B1 under high calcium conditions. Since high calcium concentrations in medium enhance osteoblast differentiation and matrix mineralization [56;57], we hypothesized that the higher CYP27B1 mRNA levels were due to an increase of the maturation state of osteoblasts [chapter 3]. Indeed, mRNA levels of DMP1 in primary human osteoblasts were also increased under high calcium conditions [chapter 3]. This is in line with a study using human trabecular bone which showed that CYP27B1 gene expression is positively correlated with gene expression of DMP1 [58]. Since DMP1 is an osteocyte marker, our 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)\textsubscript{2}D may be important for matrix mineralization which is possible under high calcium conditions. Translating the results to in vivo, an increase of CYP27B1 expression by high extracellular calcium levels is probably the result of the bone remodeling process. Bone remodeling leads to local changes in extracellular calcium concentrations due to osteoclastic bone resorption [59]. However, rats with a high dietary calcium intake show increased CYP27B1 mRNA levels compared to rats with a low dietary calcium intake [35]. Thus, the local synthesis of 1,25(OH)\textsubscript{2}D may contribute to the bone mineralization process when extracellular calcium concentrations within bone are increased due to bone remodeling or high dietary calcium intake.

The difference in regulation of CYP27B1 mRNA between bone and kidney may also answer the question as to why osteoblasts synthesize their own 1,25(OH)\textsubscript{2}D, while the kidney is able to provide 1,25(OH)\textsubscript{2}D. The local regulation of 1α-hydroxylase enables bone tissue to respond to immediate local demands. In this way, the autocrine/paracrine vitamin D system is independent of circulating 1,25(OH)\textsubscript{2}D which is regulated by systemic factors, but fulfills the local needs by local regulation [60]. When higher concentrations of 1,25(OH)\textsubscript{2}D are required within a tissue, local factors may increase the expression of CYP27B1 which in turn results in a higher availability of 1,25(OH)\textsubscript{2}D. In vitro studies also show another important difference between endogenous and exogenous sources of 1,25(OH)\textsubscript{2}D [chapter 2;10;61]. Endogenous and exogenous sources of 1,25(OH)\textsubscript{2}D have similar effects on osteoblast proliferation, differentiation and matrix mineralization in vitro [chapter 2;10;61], but endogenous 1,25(OH)\textsubscript{2}D concentrations are much lower than exogenous 1,25(OH)\textsubscript{2}D concentrations to accomplish an equal response in osteoblasts [chapter 2;10;61]. Thus it seems that endogenously synthesized 1,25(OH)\textsubscript{2}D is more efficiently utilized by differentiating osteoblasts [10;61].
Consequences of vitamin D deficiency for vitamin D metabolism in bone

Since vitamin D metabolism within bone and the effects of vitamin D on bone are local processes which are not measurable in the circulation, the use of animal models is very valuable. In this thesis, two different animal models of vitamin D deficiency were used to investigate the consequences for bone mineralization, bone structure and bone turnover as well as for local vitamin D metabolism within bone, while accompanied by adequate serum calcium and/or phosphate levels [chapter 6 and 7]. In one model, vitamin D deficiency was induced in rats within three weeks by paricalcitol injections in combination with a vitamin D deficient diet high in calcium, phosphate and lactose [chapter 6]. The other model describes long-term vitamin D deficiency in older adult mice which was induced using a vitamin D deficient diet. In both models, defects in bone mineralization and structure were not observed most likely due to sufficient calcium and phosphate in the diet. However, other factors may also contribute to the absent defects in bone mineralization and structure. In older adult mice, bone remodeling is very low with the result that little osteoid tissue may be formed and osteomalacia may not develop [chapter 7]. In addition to low bone remodeling, older adult mice may show a reduced response of bone cells to 1,25(OH)\textsubscript{2}D which may also have resulted in unchanged bone turnover and bone structure parameters compared to control animals [chapter 7]. In adult rats, the period of vitamin D deficiency (8 weeks) may be too short to result in major defects in bone mineralization or structure [chapter 6].

Results obtained using these models are in line with previously reported bone phenotype in VDR knockout animal models or animal models of dietary vitamin D deficiency [62-65], suggesting that 1,25(OH)\textsubscript{2}D signaling is not essential for bone health when hypocalcemia and hypophosphatemia are prevented. However, a reduced bone formation was observed in a few vitamin D deficiency animal models while receiving a rescue diet [66;67]. Difference in effect of vitamin D deficiency models may be explained by the duration of the experiment and age of the animals when the experiment starts [68]. Here, we used adult animals [chapter 6] or older adult animals [chapter 7] which did not show a reduction of bone formation after a period of vitamin D deficiency, but it is possible that growing animals respond differently. Studies with VDR knockout models in which a reduced bone formation was demonstrated were performed in growing animals [66;67]. Moreover, the duration of the experiment was longer in VDR knockout models showing a reduced bone formation [62;63] compared to VDR knockout models without an effect on bone formation [62;63]. This suggests that activity of 1,25(OH)\textsubscript{2}D through the VDR is required for bone formation under certain circumstances such as during growth [66;67].

In both models, CYP27B1 and CYP24 mRNA levels in bone did not change after a period of vitamin D deficiency accompanied by normal serum calcium and/or phosphate concentrations [chapter 6 and 7]. This suggests that CYP27B1 and CYP24 mRNA levels in bone are not modulated by circulating 25(OH)D or 1,25(OH)\textsubscript{2}D. Be-
cause CYP27B1 mRNA levels did not increase when serum 25(OH)D concentrations were low, the rate of conversion of 25(OH)D to 1,25(OH)$_2$D in bone cells does not change in case of vitamin D deficiency. As a result of the low amount of substrate, local 1,25(OH)$_2$D concentrations may be reduced leading to a lower or altered bone cell activity. However, a negative effect on bone structure or bone remodeling parameters was not found in the vitamin D deficiency models [chapter 6 and 7]. This suggests that, when serum calcium and phosphate levels are maintained, both low circulating and low local 1,25(OH)$_2$D levels do not result in bone abnormalities in adult animals. However, a reduced local 1,25(OH)$_2$D synthesis may have more impact on bone tissue in growing animals since CYP27B1 mRNA levels in bones from young rats (3-15 weeks) are 2-3 fold higher than in adult bone (>26 weeks) [34]. Moreover, mRNA levels of CYP24 and VDR are also higher in young animals compared to adult animals [34]. The higher expression of genes involved in vitamin D metabolism in bone tissue from young animal suggests that local vitamin D metabolism is at least important during the process of bone growth [34]. Thus, although a reduced local 1,25(OH)$_2$D synthesis may not have a negative impact on adult bone of normocalcemic animals, the consequences of a reduced local 1,25(OH)$_2$D synthesis in bones of growing animals remains to be established.

Our models using adult or older adult animals show that low circulating 1,25(OH)$_2$D levels and possibly low local 1,25(OH)$_2$D levels in bone do not have a negative impact on adult bone health in the presence of normal calcium and phosphate homeostasis. However, in certain conditions such as bone growth, 1,25(OH)$_2$D signaling may be necessary for optimal bone cell activity in the presence of normal calcium and phosphate homeostasis. Indeed, direct effects of the 1,25(OH)$_2$D/VDR system on bone were shown using conditional VDR knockout and transgenic mouse models, although effects were determined by the osteoblastic differentiation stage [69-72]. In normocalcemic mice, VDR ablation in immature osteoblasts resulted in an increased bone mass due to a reduced bone resorption [72]. Transgenic mice which over-express the VDR in mature osteoblasts showed an increased bone volume due to an increased osteoblast activity and a reduced bone resorption [69;71]. Studies using conditional VDR knockout mice and global VDR knockout mice also show that the primary role of 1,25(OH)$_2$D is to maintain an adequate calcium homeostasis which has priority over maintaining skeletal integrity [29;70;73;74]. During a negative calcium balance, serum 1,25(OH)$_2$D levels increase by the actions of PTH leading to a stimulation of intestinal calcium absorption as well as renal calcium reabsorption and an increase of the flow of calcium from bone to blood [29;74]. In bone, 1,25(OH)$_2$D increases RANKL expression in osteoblasts which subsequently leads to an enhancement of osteoclastogenesis and bone resorption [29;74]. 1,25(OH)$_2$D can also inhibit bone mineralization by upregulating levels of mineralization inhibitors [70;73].
VITAMIN D METABOLISM IN SKELETAL MUSCLE

Signaling of $1,25(\text{OH})_2\text{D}$ in skeletal muscle

Severe vitamin D deficiency in humans is accompanied by proximal muscle weakness which is predominantly caused by fast-twitch muscle fiber atrophy [75;76]. Whether muscle weakness in severely vitamin D deficient patients is caused by low serum $1,25(\text{OH})_2\text{D}$ levels is doubtful since the presence of the VDR in skeletal muscle has been disputed [77-82]. Conflicting studies with respect to detection of the VDR are due to factors such as low VDR expression in mature muscle, differences in specificity of antibodies and different protein extraction methods [77;78;80;83]. However, presence of the VDR has been clearly demonstrated in mouse skeletal muscle using a highly specific antibody and a hyperosmolar lysis buffer [79]. This study also showed that VDR expression in skeletal muscle is lower compared to that in duodenum and decreases with aging [79]. Supportive for the presence of the VDR in skeletal muscle is a VDR ablation model in which a 20% reduction of muscle fiber diameter was observed [84]. Moreover, muscle fibers were more variable in size compared to wild-type mice [84]. These muscle abnormalities were also observed in VDR ablated mice on a diet high in calcium, phosphate and lactose, indicating a direct role for $1,25(\text{OH})_2\text{D}$ in muscle [84]. To clarify the underlying mechanisms of a $1,25(\text{OH})_2\text{D}$ effect on skeletal muscle, we used a skeletal muscle cell culture model. In chapter 5, we show that in mouse C2C12 myoblasts and myotubes, VDR mRNA levels were stimulated by treatment with $1,25(\text{OH})_2\text{D}$ which suggests an increased genomic transcriptional response of skeletal muscle cells to $1,25(\text{OH})_2\text{D}$.

Activities of $1,25(\text{OH})_2\text{D}$ in C2C12 myoblasts include inhibition of cell proliferation which is consistent with their effects on osteoblasts [chapter 2 and 5]. With respect to myoblast differentiation, the effects of $1,25(\text{OH})_2\text{D}$ were small compared to those reported in other in vitro studies which can be explained by the differences in experimental setup [chapter 5]. In C2C12 myotubes, MHC mRNA levels were increased by high concentrations of $1,25(\text{OH})_2\text{D}_3$, but this did not induce hypertrophy, change expression of myogenic regulatory factors or activate the Akt/mTOR signaling pathway [chapter 5]. These results indicate that when effects also apply to in vivo muscle, effects of $1,25(\text{OH})_2\text{D}$ on skeletal muscle may 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.

When $1,25(\text{OH})_2\text{D}$ signaling does not stimulate myotube hypertrophy in vitro [chapter 5] or in vivo [85], the question arises whether muscle weakness in severe vitamin D deficient patients is due to low serum $1,25(\text{OH})_2\text{D}$ levels. Hypovitaminosis D-induced alterations in serum calcium, phosphate and PTH levels may also contribute to the development of muscle weakness during severe vitamin D deficiency [86;87]. Support comes from a rat model in which vitamin D supplementation in vitamin D deficient animals maintained muscle protein turnover by preventing hypoc-
alcemia [86]. Another rat model showed that hypophosphatemia was responsible for the observed muscle weakness during vitamin D deficiency [87]. The hypothesis that muscle weakness rather is an indirect effect of 1,25(OH)₂D is also in line with the observation that adult human skeletal muscle expresses low levels of VDR compared to skeletal muscle precursor cells [88]. These above mentioned studies suggest that secondary changes in calcium and phosphate levels may significantly contribute to muscle weakness in patients with severe vitamin D deficiency. However, low serum 1,25(OH)₂D levels may also reduce non-genomic actions of 1,25(OH)₂D such as stimulation of adenylyl cyclase/cAMP pathway, the Ca²⁺-messenger system, and MAPK cascades, which may lead to a reduced muscle function as well [89].

It can, however, be questioned whether the results in this thesis will be found in primary skeletal muscle cells, since it is known from cell lines that not all physiological functions may be retained during immortalization. For that reason, proper characterization of the cell line is necessary. Mouse C2C12 skeletal muscle cells have been shown to be an appropriate model for studying cell proliferation as well as differentiation due to their ability to differentiate in culture and to form myotubes which express myogenic proteins [90]. Moreover, our observations were consistent with those of an in vivo rat study in which supra-physiological 1,25(OH)₂D₃ levels did also not result in muscle hypertrophy [85]. Nevertheless, since differences between species (mouse versus human) and cells (cell line versus primary cells) may exist, confirmation of our results by the use of primary human skeletal muscle cells is required. In addition to the use of in vitro models, the development of a skeletal muscle-specific VDR knockout model may also help to identify the direct actions of 1,25(OH)₂D on skeletal muscle.

25(OH)D metabolism in skeletal muscle

Physical performance has been shown to associate with serum 25(OH)D concentration up to a threshold, as shown in epidemiological studies [1;8]. This suggests that 25(OH)D plays a role in skeletal muscle function. Because in osteoblasts the effects of 25(OH)D most likely occur through hydroxylation to 1,25(OH)₂D [chapter 2], we hypothesized the presence of a similar mechanism for 25(OH)D in skeletal muscle tissue. Indeed, CYP27B1 is expressed at both the mRNA and protein level in myoblasts and myotubes [12;79;91], but conversion of 25(OH)D to 1,25(OH)₂D in our C2C12 model could not be proven [chapter 5]. One reason for the absence of 1,25(OH)₂D in conditioned medium after 25(OH)D treatment may be a rapid conversion to other vitamin D metabolites. However, osteoblasts, which we used as positive control, did synthesize detectable 1,25(OH)₂D concentrations after 25(OH)D treatment. These differences in results between osteoblasts and skeletal muscle cells, may be explained by a lower activity of 1α-hydroxylase in skeletal muscle than in bone tissue [92]. In a transgenic mouse model in which DNA for the promoter region of the human CYP27B1 gene was linked to the complementary DNA for the non-mammalian luciferase, CYP27B1 promotor activity was higher in bone than in skeletal muscle [92]. Although 1α-hydrox-
ylase activity may be lower in skeletal muscle than in osteoblasts, it is possible that skeletal muscle cells enhance their 1α-hydroxylase activity when local requirements of 1,25(OH)₂D are increased, for instance during regeneration or after mechanical loading. Indeed, actively regenerating muscle shows a higher expression of CYP27B1 and VDR compared to control skeletal muscle [12]. With respect to mechanical loading, osteoblasts in culture increase their mRNA levels of CYP27B1 after mechanical stimuli [chapter 4], which may also be the case in skeletal muscle cells. An alternative explanation for the absence of 1,25(OH)₂D in conditioned medium after 25(OH)D treatment may be that the C2C12 cell line lost 1α-hydroxylase activity due to post-transcriptional abnormalities or deficient cofactors such as ferredoxin reductase or ferredoxin [chapter 5]. In that case, the production of 1,25(OH)₂D after 25(OH)D treatment needs to be demonstrated in primary human skeletal muscle cells.

When skeletal muscle cells are able to synthesize 1,25(OH)₂D from 25(OH)D, actions of 1,25(OH)₂D may be at least involved in the regulation of myoblast proliferation during development and regeneration of skeletal muscle, rather than in the regulation of muscle mass [chapter 5]. This is in line with the observation that human skeletal muscle precursor cells show a higher expression of CYP27B1 and VDR compared to adult skeletal muscle [88]. Moreover, the expression of CYP27B1 and VDR is also higher in regenerative mouse skeletal muscle than in control skeletal muscle [12]. When skeletal muscle cells are not able to synthesize 1,25(OH)₂D from 25(OH)D, the actions of 25(OH)D on C2C12 cell proliferation may be direct or via conversion to 24R,25(OH)₂D [chapter 5]. With respect to 24R,25(OH)₂D, both myoblasts and myotubes express CYP24 and respond to both 25(OH)D and 1,25(OH)₂D by strongly increased CYP24 mRNA levels [chapter 5]. Myotubes are also able to dose-dependently synthesize 24R,25(OH)₂D after 25(OH)D treatment, indicating that skeletal muscle cells are capable of preventing an excess of vitamin D metabolites within muscle tissue [chapter 5]. Whether CYP24 is regulated by other factors than 25(OH)D or 1,25(OH)₂D is unknown, but it is to be expected that CYP24 in skeletal muscle cells is regulated at a local level similar to osteoblasts. Results from experiments using osteoblast cultures also demonstrate that 24R,25(OH)₂D has stimulatory actions on osteoblast differentiation by increasing mRNA levels of alkaline phosphatase and osteocalcin [chapter 2]. Therefore, it would be of interest to investigate whether 24R,25(OH)₂D is able to affect muscle cell proliferation and differentiation.

**FINE-TUNING OF HORMONE ACTION IN TARGET TISSUES**

In the endocrine system, local metabolism of hormones within peripheral target tissues appears to be a common biological principle [93;94]. Prohormones secreted by endocrine glands or hormonal tissues can be activated by target cells resulting in local actions [93]. Cessation of local actions of the active hormone is possible by
the presence of inactivating enzymes in the target cell [93]. In this way, target cells are able to accurately regulate intracellular concentrations of the active hormone depending on their specific need at a given time [93-95]. This mechanism may be important for target tissues to fine-tune tissue responses to a specific hormone [93;94].

Local activation of prohormones in target tissues occurs especially among the group of low molecular weight, hydrophobic non-peptide hormones which exert their actions by binding to a nuclear receptor [93]. One example of a prohormone that can be activated either in the thyroid gland or peripherally is thyroxine (T4) [96]. T4 is released by the thyroid gland into the circulation and transported to a variety of target tissues such as central nervous system, brown adipose tissue and skeletal muscle [93;96]. After cellular uptake by specific membrane transporters, T4 is metabolized by iodothyronine deiodinase type 2 (D2) to the most active form triiodothyronine (T3) [97]. T3 translocates to the nucleus and binds to the TH receptor resulting in modulation of transcription of genes with TH responsive elements [97]. Inactivation of both T3 and T4 occurs by the enzyme deiodinase type 3 (D3) leading to the formation of respectively T2 and rT3 [98]. This deiodinase-mediated control of thyroid hormone signaling in target tissues depends on varying local requirements which cannot be accomplished by modulation of hormone concentrations in the circulation [99]. Serum concentrations are even unaffected when individual tissues change their thyroid hormone signaling [100]. Other examples of prohormones which can be activated peripherally include retinol, glucocorticoids, estrogens, androgens, androstenedione and steroid precursors [93;94;101].

The fine-tuning of local hormone actions in target tissues appears also applicable to the metabolism of vitamin D. In extra-renal tissues such as bone, the prohormone 25(OH)D can be converted to the most active form 1,25(OH)₂D 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)₂D are reduced by 24-hydroxylase leading to a decrease of local actions of 1,25(OH)₂D. Moreover, 1α-hydroxylase and 24-hydroxylase expression appear to be regulated at a local level in extra-renal tissues, supporting the principle of fine-tuning of hormone actions by target tissues. Since 1α-hydroxylase and 24-hydroxylase are expressed in many extra-renal tissues, the fine-tuning of local 1,25(OH)₂D concentrations within target tissues may play an important role in the vitamin D metabolism. Thus, the local synthesis of 1,25(OH)₂D and subsequently the autocrine and paracrine actions of 1,25(OH)₂D in bone and possibly in muscle are an expression of a common biological principle within the endocrine system.
CLINICAL IMPLICATIONS

**Serum 25(OH)D thresholds**
As mentioned in the general introduction, different thresholds for serum 25(OH)D exist for different health outcomes [1;8]. These differences in 25(OH)D thresholds for different tissues may be explained by the local hydroxylation of 25(OH)D to 1,25(OH)$_2$D [1]. Each tissue regulates local concentrations of 1,25(OH)$_2$D depending on its specific need which may result in different 25(OH)D thresholds for different health outcomes. The response of cells to 1,25(OH)$_2$D depends on the VDR expression and on the intracellular concentrations of 1,25(OH)$_2$D which in turn depend on expression levels of 1α-hydroxylase and 24-hydroxylase. With respect to bone and muscle, the threshold for serum 25(OH)D in relation to BMD of total hip and femoral trochanter appeared to be around the level of 50 nmol/l, while the threshold for serum 25(OH)D in relation to physical performance was around 60 nmol/l [1]. The higher threshold for serum 25(OH)D in relation to physical performance may be explained by the low expression of the VDR in adult human skeletal muscle [88]. Moreover, CYP27B1 activity may be lower in skeletal muscle compared to bone, as shown in transgenic mice [92]. Differences in 24-hydroxylase expression and activity have not been reported between bone and skeletal muscle. This suggests that due to differences in expression of VDR and 1α-hydroxylase thresholds for serum 25(OH)D may differ between bone and muscle health outcomes.

**Optimal serum 25(OH)D concentration**
The presence of local vitamin D metabolism within bone and muscle tissue has also implications for the required serum 25(OH)D concentrations in humans. After all, an adequate extra-renal synthesis of 1,25(OH)$_2$D depends not only on the level of the 1α-hydroxylase enzyme in that tissue, but also on the level of substrate [102]. However, systemic factors do not regulate CYP27B1 mRNA levels in bone [chapter 6 and 7]. As a consequence, bone CYP27B1 mRNA levels are unable to increase in case of vitamin D deficiency. This may result in diminished local 1,25(OH)$_2$D concentrations and subsequently a reduced osteoblast activity. Thus, the presence of sufficient substrate may be essential for an adequate local synthesis of 1,25(OH)$_2$D. Higher serum 25(OH)D levels may even be more advantageous, as demonstrated in vitro [chapter 2] and in vivo [1;8]. In vitro, osteoblast differentiation increases when 25(OH)D concentrations in medium increase [chapter 2]. In vivo, positive correlations exist between bone health parameters and serum 25(OH)D concentrations [1;8], although the potential role of the local synthesis of 1,25(OH)$_2$D needs to be established. Our results do not answer the question which amount of 25(OH)D is required for bone and muscle tissue to function normally, but contributes to the knowledge about the role and regulation of the local vitamin D metabolism in bone and muscle. Knowledge of both endocrine and autocrine/paracrine vitamin D metabolism is essential in the determination of the optimal serum 25(OH)D levels in humans.
Osteoporosis
Vitamin D insufficiency may result in secondary hyperparathyroidism, an increased bone turnover and bone loss leading to osteoporosis [103]. Osteoporosis is characterized by low bone mass and alteration of microstructure, leading to skeletal fragility and an increased risk for fractures [104]. Low vitamin D status may not only result in secondary hyperparathyroidism, but also to an impaired osteoblast differentiation due to reduced local 1,25(OH)\textsubscript{2}D concentrations in bone tissue which may contribute to the development of osteoporosis [105]. Thus, adequate serum 25(OH)D levels are essential for an optimal local synthesis of 1,25(OH)\textsubscript{2}D within bone tissue. Since overexpression of CYP27B1 in osteoblasts results in an increase of trabecular and cortical bone volume [55], higher 1,25(OH)\textsubscript{2}D concentrations in bone may be more advantageous, particularly in osteoporotic bone. Although more in vivo research is needed, it is possible that higher 1,25(OH)\textsubscript{2}D concentrations within bone may be obtained by relatively higher serum 25(OH)D levels [chapter 2], sufficient dietary calcium intake [chapter 3] or mechanical loading [chapter 4].

Sarcopenia
Sarcopenia is the age-related loss of skeletal muscle mass and function [106], characterized by muscle fiber atrophy, accumulation of intramuscular connective tissue and fat as well as decreased oxidative capacity [107]. In addition to the reduced function and atrophy of mature muscle fibers, sarcopenia is also accompanied by a significant decline in the function and numbers of satellite cells which may result in an impaired regenerative capacity of skeletal muscle in response to damage [107;108]. Multiple factors contribute to the development of sarcopenia including low physical activity, smoking, poor diet and age-related changes in hormone levels [106]. With aging, serum 25(OH)D levels decline [109]. Moreover, low serum 25(OH)D levels have been associated with a decreased muscle mass [110], muscle strength [111;112] and a lower physical performance [110]. This suggests that low serum 25(OH)D levels are a risk factor for the development of sarcopenia. When skeletal muscle cells are indeed able to convert 25(OH)D to 1,25(OH)\textsubscript{2}D [chapter 5], low serum 25(OH)D levels may result in a reduced local synthesis of 1,25(OH)\textsubscript{2}D. A reduced local availability of 1,25(OH)\textsubscript{2}D may have negative consequences for the process of activation and proliferation of skeletal muscle satellite cells which may contribute to the impaired regenerative capacity of sarcopenic skeletal muscle.

Chronic kidney disease
The kidney is responsible for the production of circulating 1,25(OH)\textsubscript{2}D from 25(OH)D for endocrine actions. Chronic kidney disease, characterized by a gradual loss of renal function, is associated with low serum 25(OH)D levels [113]. Reasons for low 25(OH)D levels are reduced sun exposure, decreased synthesis of vitamin D in the skin, proteinuria leading to loss of 25(OH)D bound to vitamin D binding protein (25(OH)D-DBP)
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and failure of 25(OH)D-DBP reabsorption [113;114]. Chronic kidney disease also leads to low serum 1,25(OH)₂D levels due to a reduced availability of 25(OH)D, a decrease of the renal 1α-hydroxylase availability and downregulation of renal 1α-hydroxylase expression caused by several factors such as hyperphosphatemia, increased serum FGF23 levels and uremia [113]. Subsequently, low 1,25(OH)₂D levels result in disturbed calcium and phosphate levels leading to secondary hyperparathyroidism. Due to the high prevalence of low serum 25(OH)D levels in patients with chronic kidney disease (stages 3 and 4), treatment with cholecalciferol should be initiated when serum PTH levels increase [115]. When hyperparathyreoidie continues to exist, supplementation with 1,25(OH)₂D analogues such as calcitriol or alfacalcidol should also be provided [115]. Treatment with cholecalciferol or calcidiol may also be important for the extra-renal synthesis of 1,25(OH)₂D [114]. When serum 25(OH)D levels are adequate, tissues such as bone and possibly skeletal muscle may be able to regulate local 1,25(OH)₂D concentrations depending on their need which may be required to function optimally. However, it is unclear whether the expression of 1α-hydroxylase in bone and skeletal muscle is affected by chronic kidney disease [113].

FUTURE PERSPECTIVES

This thesis provides insight into the actions of locally synthesized 1,25(OH)₂D and the regulation of local vitamin D metabolism in bone and muscle tissue. The results in this thesis have led to recommendations for future research (Fig.1).

- The local conversion of 25(OH)D to 1,25(OH)₂D has mainly been studied using in vitro osteoblast culture models [chapter 2, 3 and 4;10;11]. These models show effects of 25(OH)D, most likely via conversion to 1,25(OH)₂D, on cell growth, differentiation and mineralization of matrix [chapter 2;10;11]. However, the physiological role of the local synthesis of 1,25(OH)₂D in osteoblasts needs to be established in vivo as well. Therefore, the development of an osteoblast-specific conditional CYP27B1 knock-out model is required to prove the significance of the local synthesis of 1,25(OH)₂D within bone tissue.

- Serum 25(OH)D levels are related to bone health outcomes as well as physical performance [1;8] which may be explained by the local conversion of 25(OH)D to the most active metabolite 1,25(OH)₂D. The metabolite 25(OH)D has been considered to be inactive, but this hypothesis needs to be confirmed [chapter 2].

- In addition to osteoblasts, osteoclasts and osteocytes are also able to metabolize 25(OH)D to 1,25(OH)₂D [15;16;19]. Although effects of locally synthesized 1,25(OH)₂D on osteoblasts and osteoclasts have already been studied in vitro
[chapter 2;10;11;16;19], the role of 25(OH)D metabolism by osteocytes has not been elucidated yet. Osteocytes represent 90% of all bone cells in adult bone, and play an important role in the regulation of osteoblast and osteoclast activity [116]. Further research to determine the role of 25(OH)D metabolism within osteocytes would contribute to a better understanding of the role of locally produced 1,25(OH)₂D in bone tissue.

- Osteoblasts increase their CYP27B1 mRNA levels in response to mechanical stimuli [chapter 4]. However, an increase of 1,25(OH)₂D concentrations after 25(OH)D exposure could not be demonstrated possibly due to a rapid conversion to other metabolites or the absence of an increased 1α-hydroxylase activity [chapter 4]. Whether mechanical loading of osteoblasts, in addition to an increase of CYP27B1 mRNA levels, also results in an increased synthesis of 1,25(OH)₂D by osteoblasts, remains to be established.

- Effects of 24R,25(OH)₂D in bone tissue have not completely been elucidated so far, but several studies support a role for this metabolite in osteoblast differentiation and fracture repair [chapter 2;37;45]. Further research is therefore warranted to identify the precise role of 24R,25(OH)₂D in the process of osteoblast cell growth and differentiation. Effects of 24R,25(OH)₂D on osteoclast and osteocyte function may also be of interest to identify the function of 24R,25(OH)₂D in bone tissue.

- The conversion of 25(OH)D to 1,25(OH)₂D could not be demonstrated in the mouse C2C12 cell line possibly due to a loss of activity of 1α-hydroxylase in this cell line [chapter 5]. Therefore, local conversion of 25(OH)D to 1,25(OH)₂D in skeletal muscle cells needs to be investigated in primary human skeletal muscle cells.

- Although we could not demonstrate the presence of 25(OH)D metabolism to 1,25(OH)₂D, C2C12 myotubes were able to synthesize 24R,25(OH)₂D from 25(OH)D [chapter 5]. Osteoblast cultures and in vivo animal models, show that 24R,25(OH)₂D is able to affect osteoblast differentiation and may also play a role in fracture repair [chapter 2;37;45], suggesting that 24R,25(OH)₂D can exert actions on bone tissue. Therefore, it would be of interest to study the effects of 24R,25(OH)₂D on skeletal muscle cell proliferation and differentiation into myotubes.

- In our osteoblast culture model, we used primary human osteoblasts which display characteristics of mature osteoblasts, but immature osteoblasts may respond differently to vitamin D metabolites. The direct impact of 1,25(OH)₂D on osteoblasts depends on the osteoblastic differentiation stage [74], which is demonstrated in VDR transgenic animal models [69;71;72]. On the one hand, VDR ablation in immature osteoblasts does not alter bone formation, but results
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in a slightly increased bone mass due to reduced bone resorption [72]. On the other hand, VDR overexpression in mature osteoblasts leads to an increased bone volume due to increased osteoblast activity and reduced bone resorption [69;71]. Further research is warranted to elucidate the impact of locally synthesized 1,25(OH)₂D on immature osteoblasts.

• The effects of vitamin D metabolites on bone cell and skeletal muscle cell activity in our thesis were mostly demonstrated by the measurement of mRNA levels, but mRNA levels do not always predict protein levels [117]. After all, protein levels do not only depend on mRNA concentrations, but also on the efficiency of translation and protein degradation [117]. Next step would be the measurement of protein levels to confirm the effects of vitamin D metabolites on bone as well as skeletal muscle cell function. In addition, confirmation of changes in CYP27B1 expression by regulatory factors at the protein level is also essential as well as measurement of subsequent changes in 1,25(OH)₂D concentrations.

CONCLUSION

Osteoblasts and possibly skeletal muscle cells may be capable of fine-tuning the actions of 1,25(OH)₂D. Osteoblasts respond to 25(OH)D by a reduced proliferation and an increased expression of genes associated with osteoblast maturation and mineralization, similar to 1,25(OH)₂D. Since osteoblasts express CYP27B1 and have the ability to convert 25(OH)D to 1,25(OH)₂D, locally synthesized 1,25(OH)₂D may act in an autocrine and paracrine fashion to regulate cell growth and differentiation. Intracellular concentrations of both 25(OH)D and 1,25(OH)₂D are reduced by 24-hydroxylase which in turn leads to a decrease of local actions of 1,25(OH)₂D. Moreover, both CYP27B1 and CYP24 mRNA levels in osteoblasts appear to be regulated at a local level, and not systemically. CYP27B1 mRNA levels in osteoblasts in culture are not increased by PTH or reduced by 1,25(OH)₂D, FGF23, calcium or phosphate, as is observed in renal tubular cells. In addition, CYP27B1 mRNA levels in bone tissue are independent of circulating 25(OH)D and 1,25(OH)₂D levels. However, osteoblasts respond to mechanical stimuli by an increase of CYP27B1 mRNA levels. In this way, the local synthesis of 1,25(OH)₂D may contribute to the stimulatory effect of mechanical loading on osteoblast differentiation. Osteoblasts also respond to high extracellular calcium concentrations by increased CYP27B1 mRNA levels, which may be associated with an increased maturation state. This suggests that the local synthesis of 1,25(OH)₂D in osteoblasts contributes to the stimulatory effect of calcium on matrix mineralization. Regulation of CYP27B1 at a local level may enable bone tissue to respond to immediate local demands, independent of systemic factors. When higher concentrations of 1,25(OH)₂D are needed within a tissue, local factors may
Figure 1. Proposed mechanism of local vitamin D metabolism in osteoblasts and C2C12 skeletal muscle cells.
increase the expression of CYP27B1 which in turn may result in a higher availability of 1,25(OH)₂D. This fine-tuning mechanism of local hormone actions in peripheral target tissues appears to be a common biological principle within the endocrine system.

In addition to osteoblasts, skeletal muscle cells are also a target for 1,25(OH)₂D by affecting myoblast proliferation and increasing the expression of myosin heavy chain. In this way, 1,25(OH)₂D contributes to the process of myogenesis which is necessary for skeletal muscle regeneration and development. Similar to bone cells, skeletal muscle cells express mRNA levels of proteins involved in vitamin D metabolism. However, the synthesis of 1,25(OH)₂D from 25(OH)D in C2C12 myotubes still is a question which needs to be addressed in other models. In any case, skeletal muscle cells are able to metabolize 25(OH)D and possibly 1,25(OH)₂D by 24-hydroxylase.

The results described in this thesis provide more insight into the activity of locally synthesized 1,25(OH)₂D in bone and muscle tissue, and the factors by which local vitamin D metabolism is modulated. These results with respect to the hydroxylation of 25(OH)D to 1,25(OH)₂D in bone and possibly in muscle may explain the relationships of serum 25(OH)D levels with bone health outcomes and physical performance.

These results also increase the knowledge of the role and regulation 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 prevention and treatment of osteoporosis and other metabolic bone diseases.
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