JING-HUAN WANG

Vitamin D, Lipid Metabolism and Prostate Cancer

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ACADEMIC DISSERTATION
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To my family

"... From what I read until now I really like your thesis. Congratulations! ..."

---------- Jürgen Schauber

"... I read your thesis and liked it very much. You did a wonderful job! ... "

---------- Jürgen Schauber

CONTENTS

1. ABSTRACT

The vitamin D receptor (VDR) acts as a transcription factor upon binding of 1,25-dihydroxyvitamin D_3 (also named calcitriol) while heterodimerized with retinoic acid receptor. VDR exerts its actions through regulation of specific genes directly or indirectly. Classically, calcitriol is regarded as a regulator for the homeostasis of calcium and phosphate. Novel commonly accepted roles of calcitriol include its implication in the regulation of the immune system, brain development and neurodegenerative disorders, skin diseases and aging. Importantly, calcitriol inhibits cell proliferation, and vitamin D deficiency is associated with increased prostate cancer risk.

This study investigates the effect of vitamin D signaling on the regulation of lipid metabolism associated genes in prostate cancer cells. An interplay between the ligand for VDR, liver X receptor (LXR) and androgen receptor (AR) has been revealed. By using quantitative real-time PCR (QRT-PCR), we identified *ch25h* (cholesterol 25-hydroxylase) and *abca1* (ATP-binding cassette transporter A1) as novel calcitriol regulated genes in human primary prostate stromal and human prostate cancer LNCaP cells, respectively, with *ch25h* being up-regulated whereas *abca1* was down-regulated. CH25H is an enzyme that catalyzes the hydroxylation of cholesterol to 25-hydroxycholesterol, which in turn, inhibits cholesterol synthesis. *abca1* is a target gene of LXR. Cell growth assays indicated both of these two regulations partly contribute to calcitriol-mediated control of cell growth. Moreover, LXR agonist, TO-901317, induced the mRNA expression of 25-hydroxyvitamin D_3-24-hydroxylase (CYP24) but inhibited that of VDR in LNCaP cells. CYP24 is the most sensitive vitamin D_3 responsive gene and it is responsible for inactivation of active vitamin D metabolites such as calcitriol and calcidiol. Therefore, the studies from ABCA1 and CYP24 suggest a mutual negative regulation of the signaling downstream of their receptors by LXR agonist and VDR ligand.

The crosstalk of VDR with the other nuclear receptors, LXR and AR, is evident because calcitriol and LXR agonist regulate 17beta-hydroxysteroid dehydrogenase family enzymes (HSD17Bs). The type 2 (HSD17B2) and 4 (HSD17B4) are responsible for inactivation of active androgen. Type 5 (HSD17B5) has bi-directional effects regarding active androgen production. In the prostate it prefers to inactivate androgen where androgen plays a critical role in prostate cancer development. Our QRT-PCR study showed that androgen up-regulated type 2 and 4, but down-regulated type 5 whereas calcitriol up-regulated all three enzymes. TO-901317 down-regulated type 2 but up-regulated type 5 with no effects on type 4. The up-regulation of HSD17B2 by androgen is over 20000 fold higher than the other regulations. This implies a possible feedback loop in the prostate for local control of androgen level regulated by the VDR and LXR ligands. To further explore the role of VDR-mediated signaling on lipid metabolisms, we carried out *in vivo* studies where we found 129S1 strain VDR knockout (VDR-KO) mice, which had been on special foods containing higher calcium, had higher total serum cholesterol, but only male mice displayed a higher level of high-density lipoprotein-bound cholesterol (HDL-C). On the other hand, in the NMRI strain where wild-type (WT) mice were

switched to special foods 3 weeks before sampling, KO female mice (male not studied) had similar levels of both total serum cholesterol and HDL-C. The mRNA expression of 5 lipid metabolism associated genes from the liver tissues of 129S1 strain mice were examined by QRT-PCR. VDR-KO female mice had lower mRNA expression of SREBP2 in comparison to WT. However, VDR-KO male mice had higher levels of LXRβ and ApoAI than WT mice. We did not detect statistically significant difference regarding the expression of ABCA1 and LDLR between VDR-KO and WT mice.

Taken together, the results suggest that calcitriol/VDR is directly or indirectly involved in lipid metabolism via regulation of specific gene expression, which is implicated in the control of prostate stromal and prostate cancer cell growth. VDR, AR and LXR ligands interact and can individually control corresponding target signaling, including androgen production. It provides further information for drug discovery in the treatment of prostate cancer via VDR mediated signaling systems.

2. ABBREVIATIONS

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
1,25VD	1,25-dihydroxyvitamin D_3
β-ACTIN	beta-actin
WT	wild-type
VDR-KO	VDR knockout
VDRE	vitamin D responsive element
VDR+/-	heterozygote for VDR knockout
VDR-/-	homozygote for VDR knockout
VDR	vitamin D receptor
SREBP2	sterol regulatory element-binding protein 2
RXR	retinoic acid receptor
RPLP0	60S acidic ribosomal protein P0
PBGD	porphobilinogen deaminase
nVDRE	negative vitamin D responsive element
nVDR	nuclear VDR
LXRβ	liver X receptor beta
LXRα	liver X receptor alpha
LDLR	low density lipoprotein receptor
HSD17B5	17beta-hydroxysteroid dehydrogenases type 5
HSD17B4	17beta-hydroxysteroid dehydrogenases type 4
HSD17B2	17beta-hydroxysteroid dehydrogenases type 2
HDL-C	high-density lipoprotein-bound cholesterol
GFP	green fluorescent protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
DHT	5α-dihydrotestosterone
CYP3A4	cytochrome P450 CYP3A4
CYP2R1	vitamin D 25-hydroxylase
CYP2J3	cytochrome P450 2J3
CYP27B1	25-hydroxyvitamin D3-1-alpha-hydroxylase
CYP27A1	sterol 27-hydroxylase
CYP24	25-hydroxyvitamin D3-24-hydroxylase
CH25H	cholesterol 25-hydroxylase
calcitriol	1,25-dihydroxyvitamin D_3
calcidiol	25-hydroxyvitamin D_3
AR	androgen receptor
ApoAI	apolipoprotein AI
ABCA1	ATP-binding cassette transporter A1
$25(OH)D_3$	25-hydroxyvitamin D_3

3. LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following articles.

I **Jing-Huan Wang** and Pentti Tuohimaa (2006) Regulation of cholesterol 25-hydroxylase expression by vitamin D3 metabolites in human prostate stromal cells. Biochem Biophys Res Commun. 30;345(2):720-5.

II **Jing-Huan Wang** and Pentti Tuohimaa (2007) Regulation of 17β-hydroxysteroid dehydrogenase type 2, type 4 and type 5 by calcitriol, LXR agonist and 5α-dihydrotestosterone in human prostate cancer cells. J Steroid Biochem Mol Biol. 107(1-2):100-5.

III **Jing-Huan Wang** and Pentti Tuohimaa (2008) Calcitriol and TO-901317 Interact in Human Prostate Cancer LNCaP Cells. Gene Regulation and Systems Biology:2 97–105

IV **Jing-Huan Wang**, Tiina Keisala, Tiina Solakivi, Anna Minasyan, Allan V. Kalueff, and Pentti Tuohimaa (2009) Serum cholesterol and expression of ApoAI, LXRβ and SREBP2 in vitamin D receptor knockout mice. (Accepted to the Journal of Steroid Biochemistry & Molecular Biology)

4. INTRODUCTION

Derived from 7-dehydrocholesterol or intaken from foods, vitamin D_3 is modified further and the most hormonally active form is 1,25-dihydroxyvitamin D_3 (calcitriol). Calcitriol circulates in the blood with a limited concentration range, too high or too low will cause disease. For example, vitamin D deficiency is associated with the development of prostate cancer. Studies of vitamin D in relation to prostate cancer have been carried out for years and different mechanisms have been described. However, only few studies deal with the effects of vitamin D on lipid metabolism. Especially, during cancer cell growth large amount of lipids are needed. In our laboratory Qiao et al. found that through stimulating the expression of long-chain fatty-acid-CoA ligase 3, calcitriol inhibited fatty acid synthase expression and this played a role in the suppression of prostate cancer cell growth. Therefore, I wanted to investigate the role of vitamin D in prostate cells with lipid metabolism related aspect as a focus.

5. REVIEW OF THE LITERATURE

5.1 Vitamin D and vitamin D receptor

5.1.1 Vitamin D

Vitamin D refers to a group of compounds that have antirachitic activity. Initially it was discovered as a substance responsible for healing rickets. During the period of its discovery, it was realized that there were two antirachitic factors with distinct structures, vitamin D_2 (also known as ergocalciferol) and vitamin D_3 (cholecalciferol) (Deluca, 2005). Vitamin D_3 is the form of vitamin D that is synthesized by vertebrates, whereas vitamin D_2 is produced by plants. Both vitamin D_3 and vitamin D_2 can be further modified by corresponding enzymes to produce different vitamin D metabolites in a similar fashion. This thesis focuses on vitamin D_3. Vitamin D is not a vitamin, but rather a prosteroid hormone, not only because of when sufficient solar ultraviolet B photons are supplied our body can synthesize enough of it, but also because of the later finding that the vitamin D_3 metabolite, $25(OH)D_3$, acts more rapidly and as effectively as vitamin D_3 in healing rickets, raising blood calcium and increasing intestinal calcium transport (Horst, Reinhardt, and Reddy, 2005). We now know that the most active metabolite is $1,25(OH)_2D_3$.

Vitamin D_3 is derived from 7-dehydrocholesterol, the precursor of cholesterol, by the cleavage of the B ring of the steroid structure to form previtamin D_3 and subsequent thermal isomerization to form vitamin D_3. It seems that the best way for keeping normal plasma vitamin D_3 levels is a sunlight bath. The reason for this is that both previtamin D_3 and vitamin D_3 can absorb solar ultraviolet photons and isomerize to inactive forms with regard to active vitamin D_3 functions. When vitamin D_3 levels are high, the sunlight itself can regulate the total output of vitamin D_3 in the skin by causing the photodegradation of previtamin D_3 and vitamin D_3 (Holick, 2005). Vitamin D_3 is converted to 25-hydroxyvitamin D_3 ($25(OH)D_3$, or calcidiol) in the liver mainly by the action of 25-hydroxylase, which includes CYP27A1, CYP2R1, CYP3A4 and CYP2J3 (Prosser and Jones, 2004). It has been suggested that the production of $25(OH)D_3$ also occurred in extrahepatic tissues (Gascon-Barre, 2005), but the contribution of the extrahepatic enzymes to the circulating concentrations of $25(OH)D_3$ under normal physiological conditions is yet unknown. Under normal conditions, $25(OH)D_3$ is present at 20 to 50 ng/ml (Horst and Reinhardt, 2005a), corresponding to 12.5 to125nM. Mainly in the kidney, $25(OH)D_3$ is further modified by the enzyme 25-hydroxyvitamin D3-1-alpha-hydroxylase (CYP27B1) to result in the production of 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, or calcitriol) (Horst and Reinhardt, 2005b).

In normal human plasma, $1,25(OH)_2D_3$ circulates at around 1000 fold lower concentrations than $25(OH)D_3$ and is generally present at 20 to 65 pg/ml (Broadus et al., 1980), corresponding to 0.048 to 0.156nM. The carbon centers of $1,25(OH)_2D_3$, C-23, C-24 and C-26, are susceptible to further oxidation/hydroxylation to yield metabolites whose physiological importance is yet unclear. In general, they are considered to be catabolic in nature and play important roles in maintaining vitamin D homeostasis. By the

year of 1991, there were a total of 37 vitamin D metabolites identified (Henry and Norman, 1984; Hery and Norman, 1991).

5.1.2 Vitamin D receptor

cDNA and protein The initial cloned human vitamin D receptor (VDR) cDNA sequence presents as a transcript with two potential start sites, with one lying only three codons downstream of the first (Baker et al., 1988; Saijo et al., 1991). It is unclear whether both codons are used for initiation of translation. Interestingly, the shorter one has been shown to associate more avidly with transcription initiation factor IIB-1 (TFIIB) compared with the longer isoform (Jurutka et al., 2000). Later, transcripts with the potential to encode variant proteins with an additional 50 or 23 amino acids at the N terminus were reported (Crofts et al., 1998), which were named VDRB1 and VDRB2, respectively (Sunn et al., 2001). The original VDR (Baker et al., 1988), which is sometimes referred to as VDRA is the 48-kDa VDR isoform (Sunn et al., 2001). It has been reported that VDRB1 is expressed at a level comparable to VDRA in human tissue and cell lines (Sunn et al., 2001). An association of the VDR translation start site polymorphism and fracture risk in older women has been reported (Moffett et al., 2007). G140A in exon 3 of VDR has been revealed in three vitamin D-dependent rickets type II patients but not in normal controls (Saijo et al., 1991). The VDR belongs to the nuclear receptor superfamily, which contains 11 identified members and are included in the 48 genes for nuclear receptors according to the human genome database (Willson and Moore, 2002). Like other nuclear receptors, VDR protein makes itself a modular domain structure, which is made up of the A/B domain, the DNA-binding domain (DBD; C domain), the hinge D domain, the ligand-binding domain (LBD; E domain) and a variable F domain (Reschly and Krasowski, 2006). It was reported (Väisänen et al., 2002) that structurally different agonists have distinct ligand-receptor interactions and the amino acid residues H229, D232, E269, F279, and Y295 are critical for the agonistic conformation of the VDR.

Intracellular Trafficking Recent study using photobleaching and *in vitro* transport assays revealed that unliganded VDR and liganded VDR shuttled constantly between the cytoplasm and the nucleus (Prüfer and Barsony, 2002) and the nucleocytoplasmic exchange rate was faster for the liganded GFP-VDR (Prufer and Barsony, 2002), which indicated that the hormone can induce VDR translocation. Different protein-protein interactions explain the difference between the transport characteristics of the unliganded and the liganded VDR (Barsony and Prüfer, 2002) and retinoic acid receptor (RXR) is the most prominent candidate among all the proteins. It was hypothised (Prüfer and Barsony, 2002) that RXR was the dominant partner for the transportation of the unliganded VDR and on the other hand liganded VDR regulated the mobility and calcitriol-dependent functions of RXR. A more recent study showed that nuclear import of RXR-VDR heterodimers was mediated by VDR through its calcitriol-induced association with importinα (Yasmin et al., 2005). It seemed that VDR and RXR dimerized in the cytoplasm (Barsony, 2005) and the heterodimerization was inhibited by 9-cis-retinoic acid (9cRA) and maximally stabilized in the presence of both calcitriol and 9cRA (Yasmin et al., 2005).

There are four putative NLSs (nuclear localization signals) that reside in the VDR, designated as NLS1, NLS2, NLS3 and NLS4 in an order from N-terminus to C-terminus of the protein. Mutations in NLS1 resulted in cytoplasmic retention of both unliganded and liganded VDR. Mutations of NLS2, 3 and 4 did not cause cytoplasmic retention of the receptor in living cells (Barsony, 2005). However, mutation of NLS3 did cause calcitriol-dependent nuclear accumulation of VDR (Barsony, 2005). Apart from NLS1 and NLS3, AF2 domain was found to play a role in ligand-dependent acceleration of nuclear import of GFP-VDR (Prüfer and Barsony, 2002; Racz and Barsony, 1999). As to the nuclear export of VDR, there is evidence (Barsony, 2005) suggesting that the unliganded VDR is exported by chromosomal region maintenance 1 (Crm-1) whereas calcitriol mediated faster VDR exports is mediated by a Crm-1 independent mechanism. In addition, mutations at the calreticulin binding site in the DBD inhibited the nuclear export of GFP-VDR. Prüfer and Barsony reported that RXR dominated the nuclear import and export of the unliganded VDR (Prüfer and Barsony, 2002). Other mechanisms such as interaction of VDR with microtubule in the cytoplasm or with nuclear matrix proteins, the modifications of VDR and its coactivators by acetylation, phosphorylation etc. may also contribute the export of VDR (Barsony, 2005).

5.1.3 Mechanism of vitamin D action

5.1.3.1 Genomic action

The identified functions of vitamin D have been mainly fulfilled via its regulation of gene expression. Most of the vitamin D regulated genes are subject to an up-regulation. In these cases, VDR heterodimerizes with RXR and translocates to the nucleus, where it binds to positive vitamin D responsive element (VDRE) present on specific gene promoters. The ligand binding also plays a role in the increase of the affinity of VDR with coactivators, which act as bridges between the RXR-VDR heterodimer and the basal Pol II (DNA polymerase II) transcription machinery. VDR-RXR used to be considered as nonpermissive because they neither bind nor show activation by RXR ligand (Forman et al., 1995). However, this has been challenged. Recent studies showed 9cRA increased the formation of RXR-VDR-VDRE complex on the VDRE of osteocalcin gene to accelerate calcitriol-induced osteocalcin production in human osteoblastic cells (Jääskeläinen, Ryhänen, and Mäenpää, 2003) and 9cRA induced recruitment of coactivators by the DNA-bound heterodimer to potentiate vitamin D-dependent transcriptional responses (Sanchez-Martinez et al., 2006).

Coactivators play crucial roles in VDR mediated transcription. The identified coactivators include chromatin modeling proteins such as the SRC (steroid receptor coactivator) family and CBP/P300 (CBP stands for CREB binding protein) as well as P/CAF (P300/CBP-associated factor) coactivators, which are histone acetyltransferases that destabilize the nucleosomal core by catalyzing the acetylation of histones (Hermanson, Glass, and Rosenfeld, 2002). SKIP (ski-interacting protein/nuclear receptor coactivator-62) synergized with SRC-1 and SRC-2 to induce RXR-VDR mediated ligand-dependent transactivation by binding to VDR in its different helixes (Barry et al., 2003; Zhang et al., 2001). A ligand-dependent interaction between VDR and peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC1α) has been reported

(Savkur et al., 2005) and the same study demonstrated an intact AF-2 domain of VDR and the LXXLL motif in PGC1α were required for the coactivation of VDR transcription by PGC1α (Savkur et al., 2005).

Previously, it was reported (Rachez and Freedman, 2000) that VDR mediated transcription also occurred through a coactivator complex, termed DRIP (vitamin D receptor interacting proteins). Recently, a human multiprotein complex (WINAC) was reported to associate with VDR via one of its components, the Williams syndrome transcription factor (WSTF), to rearrange the nucleosome array around both the positive and negative VDREs and thus facilitating the coregulatory factors accessible to VDR for further transcription control (Fujiki et al., 2005; Kitagawa et al., 2003). It was also reported (Jian et al., 2005) that cyclin D3 interacted with VDR and the interaction was enhanced by calcitriol; cyclin D3 up-regulated transcriptional activity of VDR and this effect was suppressed by over-expression of CDK4 and CDK6. A novel coactivator was identified (Chen et al., 1997) and designated ACTR for nuclear receptors including VDR and it not only functioned by recruiting histone acetyltransferase (HAT) proteins CBP/P300 and P/CAF, but is also a HAT by itself. Importantly, VDR per se also directly interacts with some components of the basal transcription machinery including $TAFII_{55}$ (transcription initiation factor TFIID 55 kDa subunit) (Lavigne et al., 1999) and $TAFII_{28}$ (Mengus et al., 2000).

VDR interacted weakly with corepressor SMRT (silencing mediator of retinoid and thyroid hormone receptor) (Tagami et al., 1998). Putative corepressors which interact with VDR and recruit histone deacetylase that deacetylate the lysine residues of histone tails include NcoR-1, NcoR-2, and Hairless (Hermanson, Glass, and Rosenfeld, 2002). Alien was found to represent a new class of corepressor for VDR and it had both differences and similarities in its VDR transcription when compared to NCoR (Polly et al., 2000). As a DNA helicase (Fraser et al., 1997), SUG1 was found to interact with VDR in a calcitriol-dependent manner and this interaction has been proposed to target VDR to proteasome-mediated degradation thus to downregulate the calcitriol-activated transcriptional response (Masuyama and MacDonald, 1998). This makes the association of VDR with SUG1 a complex output concerning VDR mediated transcription and which needs further study to be understood.

Negative VDRE $1,25(OH)_2D_3$ represses the transcription of some genes such as CYP27B1 and PTH (parathyroid hormone). Efforts have been made to identify negative VDRE (nVDRE) for unravelling vitamin D mediated transcription repression. Previously, negative VDRE containing one or two repeats having nucleotide-components similarity to the up-regulatory VDRE have been reported (Demay et al., 1992; Falzon, 1996; Li and Sodek, 1993; Mackey et al., 1996). Recently, Kato S. and colleagues (Kim et al., 2007b; Murayama et al., 2004) reported a novel class of nVDRE composed of E-box-type motifs in both the CYP27B1 and PTH gene, on which VDIR (VDR-interacting repressor) binds and activates gene transcription in the absence of vitamin D. Upon ligand binding, VDR heterdimerizes with RXR and the heterodimer interacts with VDIR bound to E-box-type motifs. This causes the dissociation of P300 histone acetyltransferase co-activator from

VDIR and the subsequent association of HDAC (histone deacetylases) co-repressor complex components and thus results in ligand-induced transrepression.

Protein-protein interaction among VDR and various proteins play dominant roles in the transcriptional regulation process. In cases of positive regulation, it has been considered that in the absence of ligand binding, VDR/RXR on positive VDRE mainly associates with HDAC complex to actively repress target genes. Ligand binding leads to corepressor dissociation from VDR. Subsequently recruited coactivators have three main types of role, 1. Utilize energy from ATP hydrolysis to rearrange nucleosomal arrays; 2. Covalently modify histone tails to render specific promoter regions accessible to other coregulators; 3. Physical interaction with general transcription factors and RNA polymerase II, then transcription is started and elongated. Whereas in negative regulation, the transcription is undergoing when ligand is not bound to RXR/VDR, which is associated with coactivators and polymerase II. However, ligand binding triggers the dissociation of the coactivators and recruitment of corepressors and thus repression of transcription occurred.

Therefore, it appears that both type of VDRE mediated transcription is via co-regulator switching. The general order of proteins assembled on positive VDRE for initiation and elongation of transcription is currently not completely defined, some work has been done (Kim, Shevde, and Pike, 2005) by using real-time chromatin immunoprecipitation, which has demonstrated a suitable method for studying 1,25VD-mediated changes of chromatin organization (Väisänen et al., 2004). Interestingly, by employing this method, Väisänen et al. (Väisänen et al., 2005) demonstrated simultaneous participation of multiple, structurally diverse response elements in promoter activation in a living cell.

5.1.3.2 Epigenomic action (nongenomic action)

In addition to genomic effects of vitamin D, which usually take minutes to days to complete, some responses happen within minutes or even seconds. These rapid actions are referred to nongenomic actions of vitamin D (Falkenstein et al., 2000), which include rapid transport of intestinal Ca^{2+} (de Boland, Nemere, and Norman, 1990) and rapid increase in phosphatidylinositol-specific PLC-β activity (Schwartz et al., 2003). Also VDR and its ligands modulate kinase activity by activation of nongenotropic signals, but not transcriptional activity (Vertino et al., 2005). The mechanisms behind these phenomena are now under active study.

It has been proposed that 1,25 D_3 MARRS receptor (1,25-dihydroxyvitamin D3 membrane-associated rapid response steroid receptor), also known as Rp57/GRp58/ERp60, is a membrane version of VDR (Farach-Carson and Nemere, 2003; Kim et al., 1996; Nemere, 2005; Nemere et al., 1994; Nemere, Pietras, and Blackmore, 2003), which can bind to vitamin D and some analogue to elicit rapid responses without activation of gene transcription. A recent *in vitro* study showed that calcitriol inhibited the development of hypertrophy of chicken chondrocytes which was also mediated by MARRS (Dreier et al., 2008). Nuclear VDR (nVDR) has been found to be associated with the plasma membrane (Kim et al., 1996) or/and with caveolae in the plasma membrane (Norman et al., 2002b). Nguyen et al. reported that the nVDR is indispensable

for vitamin D mediated rapid actions (Nguyen et al., 2004). Thus, it is possible that nVDR interacts with the plasma membrane to render rapid cells response in response to its ligand. It is even possible that nVDR binds directly to ion channels and upon ligand binding the conformation of the VDR/channel complex changes, which in turn, leads to ion influx. However, it was also reported (Boyan et al., 2003) that membrane-mediated actions of vitamin D metabolites were retained in growth plate cartilage cells from nuclear vitamin D receptor knockout mice (Boston mice (Li et al., 1997)). On the other hand, truncated VDR has been shown expressed in some tissues of Tokyo strain VDR-KO mice and these receptors showed specific binding of [^{3}H]-1α,25$(OH)_2D_3$ in nuclear and caveolae membrane fractions (Bula et al., 2005). The Boston mice (Li et al., 1997) suffered a deletion of exon 3 in the VDR gene, which at the same time generated a termination codon 12 base pairs downstream and thus it is likely only a severely truncated protein with no ligand binding capability would be produced. If this is true, then there might be another protein other than nVDR which could bind vitamin D and mediate rapid actions reported in Boston mice (Boyan et al., 2003)

Norman (Norman, 2005; Norman, 2006; Norman et al., 2002a) reported that the conformation of the ligand play critical roles in eliciting the genomic or nongenomic responses. Their studies indicate that 1,25$(OH)_2D_3$ is conformationally flexible, which is an optimal agonist for both genomic and rapid responses. The 6-s-trans 1,25VD is responsible for genomic actions whereas the 6-s-cis 1,25VD is responsible for rapid nongenomic actions. There is a membrane-associated receptor that can distinguish structural differences in whether the ligand is in 6-s-cis or in 6-c-trans shape. This membrane-associated receptor might be nVDR with an alternative ligand-binding-pocket responsible for rapid actions ((Norman, 2006) and references therein).

Another type of nongenomic action of vitamin D is epigenetic action occurring in transcription events where nuclear VDR is involved. For example, 1,25$(OH)_2D_3$-induced histone deacetylation by recruited HDACs to VDR/VDIR at the nVDRE and DNA methylation at CpG sites in the promoter and exon regions of the CYP27B1 are involved in VDR mediated transcription repression (Kim et al., 2007a). Another example is that 1,25$(OH)_2D_3$ stimulates histone acetylation via coactivator recruitment by VDR on the *cyp24* promoter in intact osteoblasts (Kim, Shevde, and Pike, 2005). Importantly, impaired epigenetic mechanisms in VDR signaling are involved in malignant tumor development (Abedin et al., 2006). Nuclear receptor mediated gene regulation through chromatin remodeling and histone modifications has been reviewed in (Kishimoto et al., 2006)

5.1.4 Bile acid action on VDR

Lithocholic acid (LCA) is one of the secondary bile acids converted from bile acids that are major metabolites of cholesterol in the body. LCA is toxic and the Pregnane X receptor (PXR; NR1I2) acts as receptor for it and induces its metabolism in the liver (Staudinger et al., 2001; Xie et al., 2001). Apart from being a receptor for vitamin D_3, recent evidence indicated that VDR also bound to LCA and its derivative, 3-ketolithocholic acid (3-ketoLCA) (Makishima et al., 2002). Later it was reported LCA acetate has 30 times the potency of LCA regarding VDR activation (Adachi et al., 2005).

A very recent study (Ishizawa et al., 2008) showed that an additional LCA derivative, namely LCA propionate, induced VDR activation as effectively as LCA acetate. Both of the derivatives stimulate VDR action without causing hypercalcemia (Ishizawa et al., 2008), which has been the major side effect for vitamin D_3 derivatives in clinical application. LCA and calcitriol occupy overlapping binding sites on human VDR (Reschly and Krasowski, 2006). VDR was suggested as a bifunctional regulator, with high-affinity 'endocrine' effects mediated by calcitriol and low-affinity, detoxification effects mediated by LCA, which was exemplified by the induction of xenobiotic enzymes from the CYP3A family after VDR's binding of LCA (Jurutka et al., 2005; Reschly and Krasowski, 2006). However, it is worthy to note that activation of VDR by $1,25(OH)_2D_3$ play roles in bile acid homeostasis as well. For example, binding of calcitriol to VDRE in the rat ASBT gene stimulates both its mRNA and protein expression and enhances ileal bile acid transport (Chen et al., 2006). VDR-dependent activation of MRP3 (multidrug resistance-associated protein 3), a multispecific anion transporter for bile acids, by calcitriol and the cholestatic secondary bile acid and/or LCA has been reported (McCarthy, Li, and Sinal, 2005). Nehring et al. proposed the VDR may evolve from an original role in detoxification after considering available reports and their own identification of that substitution of vitamin D by LCA elevated serum calcium and induced CYP24 mRNA expression in the kidney of vitamin D-deficient rats (Nehring, Zierold, and DeLuca, 2007).

5.2 Functions of vitamin D

5.2.1 Classical (calcium and phosphate homeostasis)

Classically, $1,25(OH)_2D_3$ is regarded as the key regulator of Ca^{2+} (re)absorption. It enhances the active Ca^{2+} absorption in the small intestine, specifically the duodenum, and stimulates Ca^{2+} reabsorption in the kidney (Bindels, 1993; Miller and Bronner, 1981; Rasmussen et al., 1979; Wasserman et al., 1982) showing a stronger effect in the intestine rather than in the kidney of VDR-KO mice. $1,25(OH)_2D_3$, PTH and calcitonin constitute the traditional calciotropic hormones that maintain serum calcium within a narrow range by their effects on the intestine, kidney, and skeleton. In these and other calcium-transporting organs, $1,25(OH)_2D_3$ functions mainly by inducing the synthesis of related proteins. Nongenomic effects of $1,25(OH)_2D_3$ have also been reported (see section "Vitamin D and vitamin D receptor"). The level of proteins or their transcripts is known to be increased by $1,25(OH)_2D_3$ in the intestine including TRPV5 and TRPV6 (renamed after ECaC1 and ECaC2/CaT1, respectively) (Hoenderop et al., 2001; Meyer et al., 2006; Walters et al., 2007; Van Cromphaut et al., 2001; Wood, Tchack, and Taparia, 2001), which are highly selective Ca^{2+} channels and constitute the rate-limiting influx step of transepithelial Ca^{2+} transport; the intracellular calcium transfer protein calbindin-D_{9K} (Armbrecht et al., 1998; Walters et al., 2007; Van Cromphaut et al., 2001) and basolateral membrane proteins that mediate extrusion of calcium from cells, which are plasma membrane calcium ATPase ($PMCA_{1b}$) (Cai et al., 1993; Walters et al., 2007) and a sodium-calcium exchanger (Centeno et al., 2004). Reduced intestinal VDR level causes vitamin D resistance and impairs transcellular intestinal calcium absorption in mice, resulted from VDR-dependent, translational influences of $1,25(OH)_2D_3$ on calbindin D_{9k} protein production (Song and Fleet, 2007), but not TRPV6 nor calbindin D_{9k} mRNA

accumulation (Song and Fleet, 2007). The regulation of TRPV5 and TRPV6 in the intestine by vitamin D is different from regulation in the kidney of mice (Song et al., 2003). In the intestine, the PTH stimulates Ca^{2+} absorption and it is thought to be mediated by increasing the renal formation of $1,25(OH)_2D_3$, which enhances the Ca^{2+} absorption.

Vitamin D also stimulates the absorption of phosphates by stimulating the synthesis of NaPi cotransporter in small intestine (Katai et al., 1999). In the kidney, vitamin D acts synergistically with PTH to enhance calcium and phosphate resorption with more potent regulation by PTH in both cases. In the bone, the actions of vitamin D are complex, with a direct effect of mobilization of calcium out of bone. Briefly, both osteoblast and osteoclast express VDR. Upon stimulation by vitamin D, osteoblasts produce proteins such as alkaline phosphatase, collagenase, and a plasminogen activator, which are proteins for the stimulation of bone resorption. Furthermore, vitamin D and PTH promote the development of osteoclasts from precursor cells. These two processes promote bone resorption and mobilization of calcium from bone. However, the net effect of vitamin D on bone is to increase bone mineralization, which is now in general believed to be due to increasing of the calcium-phospate ion by vitamin D in plasma and extracellular fluid. It is worth noting that $1,25(OH)_2D_3$ up regulates RANKL (receptor activator of nuclear factor kappa B ligand) (Kim et al., 2006; Kitazawa, Kitazawa, and Maeda, 1999; Yasuda et al., 1998), a protein essential for osteoclastogenesis (Boyle, Simonet, and Lacey, 2003). VDRE was identified in mouse and human RANKL gene (Kim et al., 2007c). FGF23 (fibroblast growth factor 23) decreases serum P_i levels, renal Na/P_i co-transport activity and type II transporter protein levels (Inoue et al., 2005). It appears that vitamin D and FGF23 form a loop in the control of phosphate and calcium balance, in which FGF23 potentially counterbalances the actions of $1,25(OH)_2D_3$ by inhibition of 25-hydroxyvitamin D_3-1-alpha-hydroxylase and induction of CYP24 to prevent hyperphosphatemia and ectopic calcification (Shimada et al., 2004). On the other hand, synthesis of FGF23 is also strongly induced by $1,25(OH)_2D_3$ (Barthel et al., 2007).

5.2.2 Other effects (Several examples)

5.2.2.1 Cell proliferation

Current understanding of the mechanisms governing the anti-proliferative actions of calcitriol is primarily the induction of cell cycle arrest in the G_1/G_0 phase (Kobayashi et al., 1998; Ryhänen et al., 2003), due to an increase in the expression of cyclin-dependent kinase inhibitors $p21^{Waf/Cip1}$ and $p27^{Kip1}$ (Hager et al., 2001; Yang and Burnstein, 2003), a decrease in cyclin-dependent kinase 2 (Cdk2) activity (Yang and Burnstein, 2003; Zhuang and Burnstein, 1998) and the abundance of Skp1-Cullin-F-box protein/Skp2 ubiquitin ligase (Li et al., 2004), accompanied by a reduction in the nuclear fraction of this molecule and the hyperphosphorylation of the retinoblastoma protein (pRb) (Kobayashi, Hashimoto, and Yoshikawa, 1993). It has been reported that the anti-proliferative effects of $1,25(OH)_2D_3$ on breast and prostate cancer cells are associated with induction of BRCA1 (breast cancer type 1 susceptibility protein) gene expression (Campbell et al., 2000). In addition, calcitriol induces apoptosis in some cells and down-regulates some anti-apoptotic genes, like bcl-2 (Wagner et al., 2003). It is worth noting

that in circumstances when it is beneficial to the organism, vitamin D plays a role of anti-apoptosis by sharing components with the apoptosis-stimulating pathway (De Haes et al., 2004; Duque et al., 2004). The growth inhibitory action of $1,25(OH)_2D_3$ on LNCaP cells also depends on increased IGFBP-3 (insulin-like growth factor-binding protein 3) (Boyle et al., 2001), further mechanisms might involve vitamin D or/and IGFBP-3 induced $p21^{Waf/Cip1}$ expression (Boyle et al., 2001).

Other mechanisms include the stimulation of differentiation via activation of the JNK-AP1 pathway (Wang et al., 2005a; Wang, Wang, and Studzinski, 2003), modulation of growth factor actions (Trump et al., 2004), a decrease in secreted PGE_2 (prostaglandin E_2) leading to a block in cell growth stimulated by arachidonic acid and exogenous PGs (prostaglandins) (Trump et al., 2004), differential regulation of *Id1* and *Id2* genes, members of a family of transcriptional regulators for cell proliferation and differentiation (Fernandez-Garcia et al., 2005). To a wider extend, the inhibition of invasion and angiogenesis (Iseki et al., 1999) and corresponding related genes (Bao, Yao, and Lee, 2006; Ben-Shoshan et al., 2007; Pendas-Franco et al., 2008) is also involved.

5.2.2.2 Immunity

$1,25(OH)_2D_3$ suppresses adaptive immunity by inhibiting the function of antigen presenting cells (APCs). In dendritic cells (DCs), it inhibits the production of IL-12 (D'Ambrosio et al., 1998), a cytokine critical for Th1 cell development, down-regulates costimulatory molecule expression (CD40, CD80 and CD86) (Piemonti et al., 2000) and up-regulates IL-10 production (Bartels et al., 2007; Pedersen et al., 2004), thus inhibiting the development of Th1 cells. Furthermore, $1,25(OH)_2D_3$ up-regulates the expression of ILT3 (immunoglobulin-like transcript 3) (Adorini, Giarratana, and Penna, 2004), which is Ig-like inhibitory receptor and mediates inhibition of cell activation (Suciu-Foca, Manavalan, and Cortesini, 2003). However, up-regulation of ILT3 by $1,25(OH)_2D_3$ in DCs plays a role but is dispensable for its tolerogenic properties, and ILT3-independent mechanisms induced by $1,25(OH)_2D_3$ are sufficient to promote DCs to induce regulatory T cells (Penna et al., 2005).

Apart from the indirect effects mentioned above, calcitriol also has direct effects on T cells. It has been found to inhibit antigen-induced T cell proliferation (Bhalla et al., 1984; Tsoukas, Provvedini, and Manolagas, 1984) and cytokine production such as IL-2 (Alroy, Towers, and Freedman, 1995), GM-CSF (Towers and Freedman, 1998), IL-12 (Lemire, 1995) and IFNγ (Rigby, Denome, and Fanger, 1987). A direct effect of calcitriol on naïve CD4+ T cells has been shown in one study to enhance the development of Th2 cells (Boonstra et al., 2001) whereas in another to inhibit not only Th1 but also Th2 differentiation during *in vitro* polarization (Staeva-Vieira and Freedman, 2002). In addition, it induces tolerance to islet allografts via enhanced development of CD4+CD25+ regulatory cells (Gregori et al., 2001), that are suppressor T cells capable to mediate transplantation tolerance and to arrest the development of autoimmune diseases (Costantino, Baecher-Allan, and Hafler, 2008). Kong et al. demonstrated that vitamin D modulated cutaneous inflammatory reactions, at least in part, by increasing the IL-1Rα to IL-1α ratio and suppressing IL-18 synthesis in keratinocytes (Kong, Grando, and Li, 2006).

Vitamin D-mediated innate immunity has been linked to toll like receptor (TLR) after the observation (Liu et al., 2006) that TLR activation of human macrophages up-regulated the expression of VDR and CYP27B1, which resulted in the induction of the cathelicidin antimicrobial peptide (CAMP) and killing of intracellular *Mycobacterium tuberculosis* (*M. tuberculosis*). VDRE has been identified in antimicrobial peptide genes encoding CAMP (Gombart, Borregaard, and Koeffler, 2005; Wang et al., 2004) and defensin beta2 (DEFB2) (Wang et al., 2004). The induction and expression of CAMP is required for the calcitriol mediated antimicrobial activity against intracellular *M. tuberculosis* in the human monocytic cell line THP-1 (Liu et al., 2007). Interestingly, recent studies from Schauber et al. (Schauber et al., 2007) demonstrated a novel role of calcitriol in innate immunity, in which TGF-β1 and /or TLR ligands produced by keratinocytes during skin injury induced the expression of CYP27B1, which catalyzed the local production of calcitriol from calcidiol. Calcitriol, in turn, induced cathelicidin, TLR2 and CD14 to enable cells to respond to microbial stimulation. Later studies (Schauber et al., 2008) from the same laboratory identified that histone acetylation played a critical role in this function of vitamin D. The role of vitamin D in the immune response of skin has been reviewed in (Schauber and Gallo, 2008) and vitamin D directed events in the human innate immune response to *M. tuberculosis* as a physiologically relevant model system was reviewed in (Adams et al., 2007).

5.2.2.3 Neurological implication

The pleiotropic actions of vitamin D also include its being a neuroactive hormone (Kalueff and Tuohimaa, 2007; Kiraly et al., 2006; McGrath et al., 2004).

5.3 VDR deficient mice model

To date, there are four VDR-KO mice models generated by four independent research groups worldwide. The earliest and most studied models came out in 1997, which were from the groups of Marie B. Demay (Li et al., 1997)(Boston mice) and Shigeaki Kato (Yoshizawa et al., 1997)(Tokyo mice). The other two were Leuven VDR-KO mice (Van Cromphaut et al., 2001) and Erben-Germany VDR-KO mice (Erben et al., 2002). The targeted fragments of VDR in different KO mice models and the corresponding region in VDR protein was described in (Bula et al., 2005). Investigations of all four VDR-KO mice models demonstrated that the lack of functional VDR develops hypocalcemia, rickets, osteomalacia, hyperparathyroidism and alopecia. Interestingly, these abnormalities develop in mutant mice only after weaning and the mechanisms behind this remain elusive.

5.3.1 Hypocalcemia

Hypocalcemia progressively developed from day 21 along with the increase of PTH levels and hypophosphatemic (Li et al., 1997), despite the fact that PTH favors renal calcium reabsorption. By using Boston mice, Li et al. (Li et al., 2001) reported calcium reabsorption plays no significant role in the development of hypocalcemia. They demonstrated that calcium absorption as well as the urinary excretion of calcium was similar in VDR-KO and wild-type (WT) mice, but the accumulation of calcium in the serum and bone was higher in WT mice. Thus, they suggested that renal calcium

conservation was impaired in mutant mice and which contributed to the hypocalcemia in those animals. On the other hand, by using Tokyo mice and Leuven mice Crmeliet and associates (Van Cromphaut et al., 2001) showed that intestinal calcium absorption capacities were reduced in both mutant mice models. Li et al. followed a longer term when measuring the calcium absorption whereas Crmeliet et al. studied only the first 10 minutes of absorption. This may explain the discrepancy. Studies (Erben et al., 2002) using Erben-Germany mice revealed a profound renal calcium leak in normocalcemic homozygous mutants, which was reminiscent of Li's studies (Li et al., 2001). In Tokyo and Boston models, but not in Leuven mice, the intestinal and renal expression of CaBP-D9K, a vitamin D up-regulated protein involved in calcium transport, was much lower than that of corresponding WT mice, suggesting the its participation in hypocalcemia for KO mice (Li et al., 2001; Van Cromphaut et al., 2001). In contrast, VDR was not required for fetal mineral homeostasis or for the regulation of placental calcium transfer in mice (Kovacs et al., 2005).

5.3.2 Rickets

Rickets refers to a softening of the bone and it is used to describe the situation in children in comparison with osteomalacia in adults. Studies with VDR-KO mice demonstrated that it was the impairment in programmed cell death of the late hypertrophic chondrocytes which led to the characteristic findings of rickets (Donohue and Demay, 2002). In 2005, the same group (Sabbagh, Carpenter, and Demay, 2005) further demonstrated that hypophosphatemia resulted from VDR-KO impaired apoptosis of the late hypertrophic chondrocyte, which in turn resulted in rickets, through decrease of the cleavage of caspase-9.

5.3.3 Alopecia

Normalization of ionized calcium levels restored those phenotypes except for alopecia (Amling et al., 1999; Li et al., 1998), indicating VDR is required for normal hair growth. Later it was reported (Chen, Sakai, and Demay, 2001; Sakai, Kishimoto, and Demay, 2001) VDR located in epidermal keratinocytes was necessary and sufficient to prevent alopecia and the absence of a functional VDR in other cells and organs as well as the resultant metabolic phenotype did not take part into the development of alopecia. The effects of VDR concerning alopecia development were not contributed to the alteration of keratinocyte cell proliferation or differentiation, but rather to an abnormality in initiation of the hair cycle (Sakai and Demay, 2000) and/or disruption of hair follicle structure during the first catagen and thus resulting in failure of subsequent hair follicle cycling (Bikle et al., 2006). Hair grows in cycles of various phases: anagen is the growth phase; catagen is the involuting or regressing phase; and telogen, the resting or quiescent phase. Each phase has several morphologically and histologically distinguishable sub-phases. Prior to the start of cycling is a phase of follicular morphogenesis, which means the formation of the follicle. Point mutations in human VDR (hVDR) that abrogated DNA binding and RXR heterodimerization led to both rickets and alopecia. However, the effects of VDR on the hair cycle were ligand independent (Panda et al., 2001; Sakai, Kishimoto, and Demay, 2001), which was confirmed by studies in which expression of hVDR with a mutation in the ligand binding domain in mVDR-/- mice restores normal hair cycling (Skorija et al., 2005). The same study showed that a VDR transgene with a

mutation in the AF2 domain that impaired nuclear receptor coactivator recruitment resulted in a partial rescue from alopecia (Skorija et al., 2005).

Recent studies demonstrated that in keratinocytes VDR retained transactivation activities in the absence of calcitriol (Ellison, Eckert, and MacDonald, 2007). It is possible that a nuclear receptor corepressor named hairless protein (Hr) (Hsieh et al., 2003; Malloy et al., 2007) participates in VDR signal transduction in the hair cycle, in which RXR but not coactivators SRC-1 and DRIP205 is critical (Malloy et al., 2007). Hr is a transcriptional regulator known to regulate hair follicle cycling (Beaudoin et al., 2005; Panteleyev et al., 1999) and regulates VDR transcriptional activity (Xie et al., 2006) as well as interacts physically with VDR (Hsieh et al., 2003). Hr levels were greater in the skin of 19 days (catagen phase of hair cycle) old VDR-KO mice compared to WT controls (Bikle et al., 2006). Inactivating mutations in the mammalian *hr* gene resulted in hair loss in mice (Nam et al., 2006; Stoye et al., 1988) and humans (Ahmad et al., 1998; Cichon et al., 1998). Remarkably, the hair loss phenotype elicited by specific mutations in hVDR resembles the atrichia resulting from mutations in the *hr* gene (Miller et al., 2001). Recent studies (Cianferotti et al., 2007) showed that the absence of the VDR impaired canonical Wnt signaling in keratinocytes leading to a defect in keratinocyte stem cells, which in turn, caused the development of alopecia. Interestingly, Hr regulated the precise timing of Wnt signaling that is required for hair follicle regeneration (Beaudoin et al., 2005). Disruption of VDR signaling contributed to other skin disorders such as neoplasia (Zinser, Sundberg, and Welsh, 2002).

5.3.4 Fertility

VDR-KO female mice developed uterine hypoplasia in the post-weaning stage due to a lack of estrogen synthesis in the mutant ovaries (Yoshizawa et al., 1997), which has not been observed in vitamin D deficient animals. One of the consequences of hypocalcemia in KO mother mice was they conceived less often and bore smaller litters (Johnson and DeLuca, 2001; Kovacs et al., 2005). Feeding mutant mice with high levels of calcium increased the rate of conception (Johnson and DeLuca, 2001; Kovacs et al., 2005) but did not normalize the number or weight of viable fetuses (Kovacs et al., 2005). Given the fact that PTH is up-regulated significantly in vitamin D deficiency adult humans and adult VDR-KO mice, it is interesting that VDR-KO fetuses have normal serum PTH levels compared to WT and VDR+/- siblings (Kovacs et al., 2005). Serum calcitriol levels of KO mice were significantly higher than that of WT mice and were accompanied by increased 1a-hydroxylase mRNA in kidney but not placenta (Kovacs et al., 2005).

5.3.5 Renin-angiotensin system

The renin-angiotensin system is a regulatory cascade that plays an essential role in the regulation of blood pressure, electrolyte and extracellular fluid volume homeostasis. Renin expression and plasma angiotensin II production was found to be increased several fold in VDR-KO mice and the regulation was independent of calcium metabolism (Li et al., 2002). Furthermore, the transcriptional suppression of renin by calcitriol was by a VDR-mediated mechanism (Li et al., 2002), suggesting a critical role of vitamin D in electrolyte, extracellular fluid volume and blood pressure.

5.3.6 FGF23

Fibroblast growth factor-23 (FGF23), a circulating factor that plays a critical role in phosphate and vitamin D metabolism, was found undetectable after hypophosphatemia in VDR-KO mice (Yu et al., 2005). A rescue diet that normalized serum calcium and phosphorus and increased FGF23 in WT, VDR+/- and VDR-/- mice, the highest increase in VDR-/- and a medium increase in VDR+/- mice (Yu et al., 2005), suggesting that VDR is not a direct mediator of FGF23 expression, but it is critical in maintaining the balance of FGF23. On the other hand, FGF23 induced CYP24 and serum $1,25(OH)_2D_3$ levels by a VDR dependent mechanism (Inoue et al., 2005). However, FGF23 induced reduction of renal Pi transport and 25-hydroxyvitamin D_3-1-alpha-hydroxylase is VDR independent (Inoue et al., 2005).

5.3.7 Immune system

Immune cells carry VDR and individuals with severe vitamin D deficiency have immune abnormalities. The lack of functional VDR resulted in severe inflammation of the gastrointestinal tract (Froicu et al., 2003). Mathieu et al. (Mathieu et al., 2001) reported in VDR-KO mice, leukocytosis, lymphocyte subset composition in different immune organs, and splenocyte proliferation to several stimuli were normal, except for a lower response to anti-CD3 stimulation; macrophage chemotaxis was impaired but phagocytosis and killing were normal. *In vivo* rejection of allogeneic or xenogeneic islet grafts was comparable with WT mice. Low dose streptozotocin failed to induce diabetes mellitus in VDR-KO mice. Correcting hypocalcemia restored the immune abnormalities *in vitro* and the sensitivity to diabetes *in vivo*. On the other hand, treatment with $1,25(OH)_2D_3$ protected WT mice against diabetes but did not protect normocalcemic KO mice. Thus, the authors (Mathieu et al., 2001) concluded that the immune defects observed in VDR-KO mice are an indirect consequence of VDR disruption because they can be restored by calcium homeostasis normalization. More recently, it was found (Wittke et al., 2004) that VDR-KO mice with normal serum calcium level subjected to experimental induced asthma failed to develop airway inflammation, eosinophilia, or airway hyperresponsiveness, despite high IgE concentrations and elevated Th2 cytokines in contrast to WT mice under the same experimental conditions. This suggests an important role for the vitamin D endocrine system in the generation of Th2-driven inflammation in the lung. Meanwhile, it argued that it was possible that smooth muscle contractions were more sensitive to calcium perturbations due to the lack of a functional VDR.

5.3.8 Our findings

By using Tokyo model, our laboratory recently found that 25% of VDR-KO mice suffer hearing loss (Zou et al., 2008).

5.4 Vitamin D and prostate cancer

Prostate cancer (PCa) is a malignancy which is a leading cause of illness and death among men in The United States and Western Europe (Hellerstedt and Pienta, 2002; Nelson, De Marzo, and Isaacs, 2003). Being a critical factor for prostate growth, androgen promotes PCa development and thus androgen deprivation has been the most

useful therapy for treatment of PCa when surgery or radiation failed. However, many patients develop androgen-independent PCa. Thus, new methods and reagents regarding prostate cancer prevention and controlling of its progression are needed. Recently, calcitriol emerged as a promising therapeutic agent. Epidemiologic evidence supports protective roles for high plasma calcitriol levels (Wigle et al., 2008). Genetic polymorphisms of *vdr* have been associated with PCa risk (Cicek et al., 2006; Cussenot et al., 1998; Holick et al., 2007; Huang et al., 2006; Wigle et al., 2008). An association between polymorphisms of the genes for the receptors of androgen and vitamin D with prostate cancer risk was reported in a small Mexican population (Patino-Garcia et al., 2007). However, Andersson et al.(Andersson, Varenhorst, and Söderkvist, 2006) reported that *TaqI* polymorphism in the VDR gene and association for the combined AR and VDR polymorphisms was not found associated with increased prostate cancer risk. $25(OH)D_3$ in plasma interacted with the VDR *FokI* polymorphism to modify prostate cancer risk (Li et al., 2007). Interestingly, recent studies suggest an interaction between exposure to ultraviolet radiation and polymorphisms in the vitamin D receptor gene (Moon et al., 2006; Rukin et al., 2007). Prostate cancer susceptibility was mediated by sun exposure in adulthood as well as in early life protects against prostate cancer (John, Koo, and Schwartz, 2007).

By extending the observations published by Miller et al. that LNCaP cells expressed VDR (Miller et al., 1992), several studies demonstrated that $1,25(OH)_2D_3$ (Skowronski, Peehl, and Feldman, 1993) and its analogs (Bauer et al., 2003; Hedlund et al., 1997; Schwartz et al., 1994; Skowronski, Peehl, and Feldman, 1995) inhibited the proliferation and promoted the differentiation of LNCaP, PC3 and DU145 cells. These findings led to the hypothesis that vitamin D might exert beneficial actions on prostate cancer risk. The growth-inhibitory action of $1,25(OH)_2D_3$ depends on IGFBP3 up-regulation (Peehl, Krishnan, and Feldman, 2003), G1 phase cell cycle accumulation through up-regulation of the cyclin dependent kinase inhibitor p27 (p27Kip1) as well as increased association of p27Kip1 with cyclin dependent kinase 2 (Stewart and Weigel, 2004; Yang et al., 2002), induction of apoptosis (Blutt et al., 2000a) and interrupting of IL-8 signaling (Bao, Yao, and Lee, 2006). Recent report showed that in prostate cancer cells fish oil enhanced the inhibition of G1/S-phase transition through calcitriol (Istfan et al., 2007). The growth inhibition action of calcitriol did not necessarily require androgen activity (Murthy, Agoulnik, and Weigel, 2005; Yang et al., 2002) although some aspects of its actions were androgen-dependent (Zhao et al., 1997).

In addition, inhibition of prostaglandin (PG) metabolism by calcitriol attenuated growth stimulation by PG in prostate cancer cells (Moreno et al., 2006; Moreno et al., 2005). Genistein, a major component of soy, inhibited the growth of prostate cancer (Barnes, Peterson, and Coward, 1995; Kikuno et al., 2008; Lakshman et al., 2008; Majid et al., 2008) and increased the half-life of bioactive calcitriol by the inhibition of CYP24 (Farhan, Wähälä, and Cross, 2003; Swami et al., 2005). It also targeted prostaglandin pathways in the inhibition of cyclooxygenase-2 (COX-2) activity with no effects on its protein expression, thereby reducing the production of PGE_2 (Ye et al., 2004). The combination of calcitriol and genistein caused synergistic growth inhibition of PCa (Rao et al., 2002; Swami et al., 2007) and acted additively to inhibit the PG pathway (Swami

et al., 2007). Thus, it was (Krishnan et al., 2007b; Swami et al., 2007) postulated that the combination of calcitriol and genistein was a therapeutic option for the treatment of PCa. The combination of calcitriol with S179D Prolactin (PRL) (Wu, Zanello, and Walker, 2007), cetuximab (Belochitski et al., 2007) and non-steroidal anti-inflammatory drugs (NSAIDs) such as NS398, SC-58125, naproxen and ibuprofen (Krishnan et al., 2007a) and the co-treatment with 4-HPR and cholecalciferol (Tokar et al., 2006), was also suggested for the treatment of PCa.

Other mechanisms for calcitriol in anti-proliferation of prostate cancer cells include through down-regulation the expression of PTHrP (Shen et al., 2007), hypoxia-inducible factor (HIF)-1 and its target genes such as the vascular endothelial growth factor (VEGF) (Ben-Shoshan et al., 2007), the later impinges a role for calcitriol in inhibition of tumorigenesis and angiogenesis. 25-hydroxyvitamin D_3-3-bromoacetate (25-OH-D_3-3-BE), a derivative of 25-hydroxyvitamin D_3, has strong growth-inhibitory and proapoptotic properties in hormone-sensitive and hormone-refractory prostate cancer cells, which is mediated by VDR and is related to the inhibition of phosphorylation of protein kinase B (AKT or PKB) (Lambert et al., 2007). Our group (Qiao et al., 2003; Qiao and Tuohimaa, 2004a; Qiao and Tuohimaa, 2004b) and others (De Schrijver et al., 2003) reported that fatty acid metabolism was involved in calcitriol mediated cell proliferation. Recently, it was suggested that calcitriol had anti-inflammatory actions that may play an important role in the prevention of PCa development after the observation that calcitriol increased the expression of MAP kinase phosphatase 5 (MKP5) (Nonn et al., 2006) and Mullerian Inhibiting Substance (MIS) (Krishnan et al., 2007a), also known as anti-Mullerian hormone (AMH). The up-regulation of MKP5 was observed in primary cells derived from normal prostatic epithelium and primary, localized adenocarcinoma but not in the established PCa cell lines. In reminiscent of this, the growth inhibition of cell lines is generally reversible even after long term $1,25(OH)_2D_3$ exposure (Esquenet et al., 1996), whereas primary cultures, regardless normal or malignant, become irreversibly growth suppressed even after 2 hours of $1,25(OH)_2D_3$ treatment (Peehl et al., 1994). Interestingly, calcitriol has a radiosensitization effect on prostate cancer cells to IR (ionizing radiation) by selectively suppressing IR-mediated RelB activation, leading to a reduced expression of its target gene MnSOD (Xu et al., 2007), which is a primary antioxidant enzyme in mitochondria.

On the other hand, some other negative factors need to be taken into consideration concerning calcitriol as drug for prostate cancer therapy. For example (Kaeding et al., 2008), in prostate cancer LNCaP and 22Rv1 cells calcitriol decreases the expression and activity of the UDP-glucuronosyltransferase (UGT) 2B15 and 2B17, which are enzymes crucial in the inactivation of androgens in the human prostate. This could limit the antiproliferative properties of calcitriol in prostate cancer cells. Increased VDR corepressors NCoR1 and SMRT expression resulted in reduced VDR-mediated transcriptional activity and attenuated antiproliferative response to vitamin D in prostate cancer cells (Abedin et al., 2006; Khanim et al., 2004; Ting et al., 2007). Calcitriol enhances the proliferation of rat prostate cancer cells in the presence of living bone although exerts inhibition effect when treated alone (Herring et al., 2007). Therefore, the efficacy of vitamin D based treatment involves the whole vitamin D/VDR mediated

signaling system and different critical aspects need to be considered simultaneously during vitamin D-based drug discovery and therapy.

In vivo, treatment of mice with $1,25(OH)_2D_3$ reduced both of the size and the weight of tumors in PC-3 and LNCaP xenograft models (Blutt et al., 2000b; Gross, Peehl, and Feldman, 1997; Polek et al., 2001; Schwartz et al., 1995). It was also reported that $1,25(OH)_2D_3$ inhibited the invasiveness of prostate cancer cells presumably by decreased expression of the matrix metalloproteinases MMP-2 and MMP-9 (Schwartz et al., 1997) and of the expression of α_6 and β_4 integrins (Sung and Feldman, 2000). A recent study (Banach-Petrosky et al., 2006) showed that calcitriol inhibited the formation of PIN in *Nkx3.1; Pten* mutant mice, and it was maximally effective when delivered before the occurrence of PIN or invasive cancer, which supported the assessment of vitamin D analogues in clinical trials of patients who were diagnosed with PIN or before the development of PIN (Banach-Petrosky et al., 2006).

To evaluate the safety and efficacy of treatment of prostate cancer with calcitriol or its analogs, alone or in combination with other chemicals, clinical trials have been carried out in PCa patients (Beer, 2005; Beer et al., 2005; Beer and Myrthue, 2004; Beer et al., 2008; Beer et al., 2007; Flaig et al., 2006; Petrioli et al., 2007; Schwartz et al., 2005; Tiffany et al., 2005; Trump et al., 2006a; Trump et al., 2006b).

5.5 Vitamin D and lipid metabolism

Derived from cholesterol, vitamin Ds are characterized by cleavage of the B ring of the core structure, hence the "seco" prefix Vitamin D (Jones, Strugnell, and DeLuca, 1998). Various forms of vitamin Ds belong to sterol-class of lipids (Fahy et al., 2005). Increasing evidence suggest that the vitamin D endocrine system is related to obesity, which has been found to be associated with lower levels of serum 25(OH)D (Arunabh et al., 2003; Bell et al., 1985; Buffington et al., 1993; Chiu et al., 2004; Liel et al., 1988; Need et al., 1993; Parikh et al., 2004; Stein et al., 2001; Vilarrasa et al., 2007; Wortsman et al., 2000) and serum $1,25(OH)_2D_3$ (Parikh et al., 2004). A strong inverse association between total body fatness and serum calcidiol levels has been reported (Hahn et al., 2006; Snijder et al., 2005) and this relationship is independent of age, sex, season, study region, smoking and race (McKinney, Breitkopf, and Berenson, 2008; Snijder et al., 2005). Supplementation of vitamin D resulted in weight loss (Lind et al., 1989; Ljunghall et al., 1987; Major et al., 2007)..

The reduction in serum 25(OH)D with increased adiposity has been assumed to be due to enhanced sequestration of vitamin D in fat (Blum et al., 2008; Holick, 2007; Liel et al., 1988; Wortsman et al., 2000). In agreement with this, Reinehr et al. (Reinehr et al., 2007) reported that the significantly higher PTH and lower 25(OH)D concentrations in obese children were normalized after weight loss, suggesting that the changes are consequences rather than causes of overweight. Another mechanism concerning vitamin D and obesity is that low vitamin D_3 may impair insulin action, glucose metabolism and various other metabolic processes in adipose and lean tissue (McCarty and Thomas, 2003; McGill et al., 2008). Interestingly, a survey (Kamycheva, Joakimsen, and Jorde, 2003) based on the questionare of life-style factors, with a special emphasis on calcium and vitamin D

intakes from men (*n* = 9252) and women (*n* = 9662) indicated that calcium and vitamin D intakes may have opposing effects on body weight. In addition, Ortega et al. (Ortega et al., 2008) reported that women (20 - 35 years) with a better vitamin D status respond more positively to hypocaloric diets and lose more body fat. The later two studies support the second mechanism, which is vitamin D_3 may has effects on body fat via regulation of insulin action, glucose metabolism and various other metabolic processes in adipose and lean tissue (McCarty and Thomas, 2003; McGill et al., 2008). In addition, the relationship consistently observed between calcidiol and body composition is not due to adaptation to changes in transport proteins, DBP (vitamin D binding protein) (Bolland et al., 2007) and reduced sun exposure, at least in older adults (≥ 65y) (Harris and Dawson-Hughes, 2007).

In obese individuals, concurrence of increased PTH levels, decreased vitamin D and metabolic syndrome have been frequently found. Studies regarding the relationship among these three characters have been reported, but have not been consisted. In comparison with vitamin D, serum PTH level is positively associated with the body mass index (Kamycheva, Sundsfjord, and Jorde, 2004). One hypothesis is that the physiologic increase in PTH levels in response to hypovitaminosis D increases intracellular calcium in adipocytes, which leads to increased lipogenesis and weight gain (McCarty and Thomas, 2003). On the other hand, obese children had significantly higher PTH and lower calcidiol concentrations but a relationship between PTH, vitamin D, and insulin sensitivity was not found (Reinehr et al., 2007). In general, studies suggest that vitamin D deficiency is associated with the metabolic syndrome (MS) (Botella-Carretero et al., 2007; Hahn et al., 2006; Manco et al., 2005) and PTH level (Hahn et al., 2006) in obese patients, and do not support an independent contribution of 25(OH)D nor PTH in the pathogenesis of the MS in obese subjects (Rueda et al., 2008).

At molecular level, studies of vitamin D on lipid metabolism associated genes or their products provide evidence and ways for understanding the mechanism of vitamin D in relation to lipids. Significant findings include the identification of VDRE in the promoters of genes encoding human peroxisome proliferator-activated receptor delta (PPARδ) (Dunlop et al., 2005), mice insulin-induced gene 2 protein (Insig2) (Lee et al., 2005) and human apolipoprotein A-I (ApoAI) (Wehmeier et al., 2005). Recent studies, using the mouse 3T3-L1 preadipocyte (Kong and Li, 2006) and porcine preadipocyte (Zhuang, Lin, and Yang, 2007) cell culture models, have shown that vitamin D inhibits adipogenesis. This is mediated through a VDR-dependent inhibition of CCAAT/enhancer-binding protein alpha (C/EBPα) and PPARγ expression (Kong and Li, 2006), a decrease in PPARγ mediated transactivation activity (Kong and Li, 2006), and decreased porcine preadipocyte differentiation, down-regulation of the expression of adipogenesis-related genes resulted from suppression of PPARγ and RXRα mRNA expressions (Zhuang, Lin, and Yang, 2007). An interaction of vitamin D and a short chain fatty acid, butyrate, demonstrated that differentiation and cell cycle arrest of Caco-2 cells induced by butyrate were mediated by up-regulation of 25-hydroxyvitamin D_3-1-alpha-hydroxylase protein and enzymatic activity (Schroder et al., 2005), and VDR (Gaschott and Stein, 2003; Gaschott et al., 2001) in which p38-MAPK was involved (Daniel et al., 2004). Qiao et al. (Qiao et al., 2003) reported that inhibition of fatty acid

synthetase expression by calcitriol in prostate cancer cells was through stimulating the expression of long-chain fatty-acid-CoA ligase 3 (Qiao and Tuohimaa, 2004a; Qiao and Tuohimaa, 2004b). In duodenal cells, PTH and calcitriol activate a Ca^{2+} dependent cytosolic PLA2 (phospholipases A2) and subsequent arachidonic acid release partly as a result of ERK1/2 pathway stimulation (Gentili, Morelli, and de Boland, 2004). Sakuma et al. suggested a potential of VDR to couple with PPAR signaling pathway for regulating of lipid metabolism (Sakuma et al., 2003). Series studies from Zemel MB et al., suggested that suppression of calcitriol level by high calcium inhibits adipocyte UCP2 (mitochondrial uncoupling protein 2) expression and have an effect on anti-obesity (Shi, Dirienzo, and Zemel, 2001; Shi et al., 2002; Sun and Zemel, 2004; Zemel et al., 2000).

Heterozygous and homozygous VDR mutant mice showed reduced body weight throughout life since three weeks old (Erben et al., 2002; Li et al., 1997). Further studies by using VDR knockout mice are needed.

5.6 Interaction of VDR and other signaling systems

VDR belongs to the class II nuclear receptor subfamily, which also includes retinoic acid receptors (RARs), retinoid X receptors (RXRs), thyroid hormone receptors (TRs), peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs), and the farnesoid X receptor (FXR). **RXR** mediates the functions of VDR and the other receptors mentioned here by forming a heterodimer with each of them. The association between VDR and RXR has been well known (Davidenko et al., 1995; Jin et al., 1996; Mangelsdorf et al., 1995).

The formation of VDR-**TR** heterodimer has been reported (Schrader et al., 1994). However, it was not supported by later studies (Raval-Pandya et al., 1998; Yen et al., 1996). On the other hand, these authors proposed that cross-talk between the VDR- and TR-signaling pathways was due to the formation of VDR/RXR heterodimer on TREs (thyroid hormone response elements) (Yen et al., 1996) and/or sequestration of RXR by TR (Raval-Pandya et al., 1998). Consistent with the observation of the negative activity of VDR on TR-mediated transcriptional activity in (Raval-Pandya et al., 1998; Yen et al., 1996), an earlier study (Kaji and Hinkle, 1989) showed calcitriol down-regulated nuclear thyroid hormone receptors and diminished growth hormone induction by triiodothyronine (T_3). In the process of adipocyte differentiation, calcitriol and T_3 primarily acted synergistically but then antagonically at enhanced concentrations (Dace et al., 1997; Lenoir et al., 1996), this may result from transient up-regulation of the expression of the *TRß* gene by physiological (low) concentration of calcitriol and cross-downmodulation of the respective receptor sites by the two reagents (Schneider et al., 2005). Calcitriol inhibits thyroid hormone-induced osteocalcin (Varga, Spitzer, and Klaushofer, 2004; Varga et al., 2003) and a thyroid hormone response element is required (Varga et al., 2003).

Liganded VDR inhibits the transativation by **PPARα**/clofibric acid (Sakuma et al., 2003) and **FXR**/chenodeoxycholic acid (CDCA) (Honjo et al., 2006), in both cases LBD of VDR is needed, but not DBD (Honjo et al., 2006; Sakuma et al., 2003). Direct interaction of FXR with VDR may play a role in the transcriptional inhibition (Honjo et al., 2006).

Possible involvement of **SXR** (SXR; also known as pregnane X receptor PXR, PAR, and NR1I2) in enhanced CYP24 expression in drug-induced osteomalacia has been described (Pascussi et al., 2005), and was argued by a later study (Zhou et al., 2006), which demonstrated that SXR played a dual role in mediating vitamin D catabolism and drug-induced osteomalacia, by activation of CYP3A4 and inhibition of CYP24. CYP3A4, along with CYP2C9 and CYP2B6 has been found to be regulated by VDR (Drocourt et al., 2002), SXR (Gerbal-Chaloin et al., 2001; Goodwin et al., 2001; Pascussi et al., 2003) and **CAR** (Gerbal-Chaloin et al., 2002; Goodwin et al., 2002; Pascussi et al., 2003; Sueyoshi et al., 1999). Shared response elements of VDR/RXR, SXR/RXR and CAR/RXR have been reported to be involved in CYP2B6, CYP2C9 and CYP3A4 gene regulation (Drocourt et al., 2002). Furthermore, VDR-mediated CYP3A4 reporter gene induction was inhibited by PXR or CAR co-transfection (Drocourt et al., 2002). CYP2C9 was also transactivated by human **glucocorticoid receptor** (Gerbal-Chaloin et al., 2002).

Calcitriol directly up-regulates **PPARδ** (Dunlop et al., 2005). In addition to sharing the ability of RXR binding, FXR, SXR, and VDR are all bile acid receptors that regulate bile acid metabolism (Makishima, 2005). This not only implies the interactions between FXR, SXR and VDR, but also suggests a cross-talk of their actions with the **LXR** mediated pathway, which has been raised recently after the observation of VDR in the inhibition of LXRβ signaling (Jiang et al., 2006).

Calcitriol induces the expression of **AR** in LNCaP human prostate cancer cells (Zhao et al., 1999) and some of its actions in LNCaP human prostate cancer cells are androgen-dependent (Zhao et al., 1997). Up-regulation of AR by $1,25(OH)_2D_3$ and of VDR by 5α-dihydrotestosterone (DHT) have been observed in OVCAR-3 cells (Ahonen et al., 2000). The same study showed that $1,25(OH)_2D_3$ inhibited AR stimulated cell growth (Ahonen et al., 2000). Another study demonstrated that in PC3 and LNCaP cells, AR suppressed VDR transactivation by competition for shared coregulator ARA70 (70 kDa androgen receptor coactivator) (Ting et al., 2005). DHT interacted with $1,25(OH)_2D_3$ in the regulation of chondrocyte proliferation and differentiation (Krohn et al., 2003), and increased VDR DNA binding activities of normal human prostate epithelial cell 267B-1 and stromal cells in the presence of $1,25(OH)_2D_3$ (Leman et al., 2003b). In 267B-1 cells, $1,25(OH)_2D_3$ upregulated the AR protein level in both the cytoplasmic and nuclear fractions and increased the AR DNA binding activities in the nuclear fractions (Leman and Getzenberg, 2003). In *in vivo* study, 3 week treatment with $1,25(OH)_2D_3$ of rats decreased the prostatic size by 40% in non-castrated animals but had no influence on the size of the prostate in castrated rats (Leman et al., 2003a). Interestingly, $1,25(OH)_2D_3$ increased the AR protein levels in both intact and castrated groups (Leman et al., 2003a).

Taken together, the cross-talks between VDR and other nuclear receptors mediated signaling systems exist in various biological processes. The mechanisms behind this process seem to be complicated, which include direct or indirect protein-protein interactions of VDR with other nuclear receptors, occupation of NRRE (nuclear receptor responding element) by VDR/RXR, competition of co-factors for transcription, common regulation of genes, cross-modulation of the level of agonist(s) by regulation of corresponding related enzyme(s) and competition or interfering of NRRE binding.

5.7 Lipid metabolism associated genes

5.7.1 CH25H and SREBP2

The gene for human cholesterol 25-hydroxylase (CH25H) is located on chromosome 10q23 and it expands one exon (Lund et al., 1998), whose protein product is an enzyme involved in cholesterol metabolism. CH25H belongs to a family of enzymes that utilize di-iron cofactors to catalyze the hydroxylation of hydrophobic substrates such as cholesterol to 25-hydroxycholesterol (Lund et al., 1998). 25-hydroxycholesterol is one of the natural oxysterols (Schroepfer, 2000), and inhibits the activation of sterol regulatory element binding proteins (SREBPs) (Adams et al., 2004), which are transcription factors (Wang et al., 1994) that regulate genes involved in the synthesis of cholesterol and other lipids in animal cells (Brown and Goldstein, 1999; Ericsson et al., 1996; Goldstein and Brown, 1990; Guan et al., 1995; Osborne, 1995; Yokoyama et al., 1993).

SREBPs are synthesized as precursors complexed with SREBP cleavage-activating protein (SCAP) in the membrane of the endoplasmic reticulum (ER) (Brown and Goldstein, 1999). SCAP is a dual functional protein by being both an escort protein and a sterol sensor. When cells are depleted of sterols, SCAP escorts SREBPs from ER to Golgi where the SREBPs are cleaved sequentially by two proteases (Hua et al., 1996; Sakai et al., 1996). Cleavage releases the bHLH-Zip domain which travels to the nucleus where it activates genes whose products play roles in the lipid synthesis and uptake (Yang et al., 1994), including cholesterol (Horton et al., 2003). Cholesterol has been shown to directly bind to the sterol-sensing domain of SCAP *in vitro* (Radhakrishnan et al., 2004). The binding of cholesterol to SCAP elicits a conformational change in SCAP, which causes SCAP to bind to Insigs (insulin-induced genes), which are endoplasmic reticulum retention proteins that abrogate movement of the SCAP.SREBP complexes to the Golgi apparatus for SREBP processing (Adams, Goldstein, and Brown, 2003; Brown et al., 2002; Feramisco et al., 2005; Radhakrishnan et al., 2004). 25-hydroxycholesterol appears to be a more potent mediator in eliciting SCAP binding to Insigs (Adams et al., 2004), although without causing a detectable confirmation change in SCAP (Adams et al., 2004).

SREBP inhibition leads to reduced cholesterol synthesis. In line with this, numerous data indicates that 25-hydroxycholesterol represses cholesterol synthesis in various cell types (Cerda, Wilkinson, and Broitman, 1995; Russell, 2000). Additionally, 25-hydroxycholesterol acts as an agonist for LXR to activate LXR targeted gene transcription (Venkateswaran et al., 2000). Furthermore, 25-hydroxycholesterol seems to inhibit the growth of both tumour and normal cells (Larsson and Zetterberg, 1995; Zhou et al., 2004) and induces apoptosis through the inhibition of c-myc (Ayala-Torres, Zhou, and Thompson, 1999).

5.7.2 LXR and ABCA1

The LXRs were first identified as orphan nuclear receptors (Willy and Mangelsdorf, 1998) and later they were shown to be activated by a specific class of naturally occurring oxysterols derived from cholesterol (Fu et al., 2001; Janowski et al., 1996; Lehmann et al., 1997). LXRs function as heterodimers with RXR to regulate gene expression (Laffitte

et al., 2001; Mangelsdorf et al., 1995; Repa and Mangelsdorf, 2002; Repa et al., 2000; Willy et al., 1995). In mammals there are two forms of LXRs, LXRα/NR1H3 and LXRβ/NR1H2. The expression of LXRα is restricted to kidney, intestine, spleen, and adrenals, with the highest level of expression in the liver (Apfel et al., 1994; Willy et al., 1995) whereas LXRβ is ubiquitously expressed (Shinar et al., 1994; Song et al., 1994).

Many of the target genes for LXRs are involved in cholesterol and fatty acid metabolism pathways (Lehmann et al., 1997; Luo and Tall, 2000; Schultz et al., 2000) and thus LXRs function as cholesterol sensors, and regulators of genes for cholesterol efflux, bile acid production, and lipid transportation to maintain cholesterol homeostasis (Naik et al., 2006; Repa and Mangelsdorf, 2000; Tangirala et al., 2002; Tontonoz and Mangelsdorf, 2003). Cholesterol and bile acid metabolisms are impaired in mice lacking the nuclear oxysterol receptor LXRα (Peet et al., 1998). Major cholesterol-related targets of LXRs include ATP-binding cassette transporter family members such as ABCA1 (Costet et al., 2000; Repa et al., 2000), ABCG1 (Kennedy et al., 2001), ABCG5 (Berge et al., 2000) and ABCG8 (Repa et al., 2002).

The human *abca1* gene was assigned to chromosome 9q31, spanning a minimum of 70 kilo-bases and containing at least 49 exons (Brousseau et al., 2000; Luciani et al., 1994; Remaley et al., 1999). It is mutated in Tangier disease (Brooks-Wilson et al., 1999; Rust et al., 1999), which in turn, is featured by low or absence of HDL-C and reduced total cholesterol (Serfaty-Lacrosniere et al., 1994) with increased susceptibility to atherosclerosis (Maxfield and Tabas, 2005). Interestingly, recent studies show that the LXR agonist inhibits tumor growth and progression of LNCaP prostate cancer cells (Chuu et al., 2006; Chuu et al., 2007; Fukuchi et al., 2004b), and androgenic inhibition of ABCA1 is involved in the regulation of prostate cancer growth (Fukuchi et al., 2004a).

5.7.3 HSD17B2, HSD17B4 and HSD17B5

17beta-hydroxysteroid dehydrogenases (HSD17Bs) are a group of enzymes that are involved in interconversion of active and inactive forms of androgens and estrogens (Brereton et al., 2001; Geissler et al., 1994; He et al., 1999; He et al., 2001; Luu The et al., 1989; Song et al., 2006; Torn et al., 2003). HSD17B2 was identified as a membrane-bound enzyme which preferentially catalyzes the oxidation of the 17β-hydrxyl group of estradiol-17β and testosterone as well as the 20α-hydroxyl group of 20α-dihydroprogesterone (Blomquist, Lindemann, and Hakanson, 1985). HSD17B2 is expressed in breast, uterus, testis, liver, and prostate tissues but it is expressed especially high in placenta tissue (Casey, MacDonald, and Andersson, 1994; Moghrabi, Head, and Andersson, 1997; Peltoketo, Vihko, and Vihko, 1999; Wu et al., 1993). Estradiol, progesterone and activin have been reported to regulate the expression of 17beta-hydroxysteroid dehydrogenases (Blomquist et al., 1995; Ghersevich et al., 2000; Poutanen et al., 1990). Hughes et al. (Hughes et al., 1997) found that calcitriol up-regulated HSD17B2 at both the mRNA and enzyme activity levels in cultured keratinocytes. An association of the loss of heterozygosity at HSD17B2 gene containing chromosomal region, 16q24.1-16q24.2, and a risk for clinically aggressive prostate cancer has been reported (Casey, MacDonald, and Andersson, 1994; Elo et al., 1997; Elo et al., 1999). The expression level of HSD17B2 is significantly higher in benign prostatic

hyperplasia compared with the carcinoma specimens (Elo et al., 1996). During the cellular transformation of LNCaP prostate cancer cells from the androgen dependent stage to the androgen independent stage the relative expression of HSD17B2 decreased markedly (Härkönen et al., 2003).

hsd17b4 is located at chromosome 5q2 and its protein product is ubiquitously expressed in a wide variety of tissues and catalyses oxidative reactions such as conversion of testosterone into androstenedione (Penning, 2003). It was reported that HSD17B4 expression was abolished after vitamin D-propagated differentiation in human THP1 myeloid leukemia cells (Jakob, Homann, and Adamski, 1995). Human primary ovarian surface epithelium (hOSE) cells express HSD17B4 mRNA but not HSD17B2 mRNA. HSD17B4 is suggested to be involved in estrogen inactivation in these cells (Nagayoshi et al., 2005).

HSD17B5 (AKR1C3) is expressed in the liver, endometrium, ovary, prostate and mammary gland (Luu-The et al., 2001; Penning et al., 2001). It acts as a 17-ketosteroid reductase, converting androstenedione into testosterone. In the prostate the enzyme seems to prefer DHT and androstenedione as substrates and thus favors the inactivation of highly active DHT (Penning et al., 2001).

5.7.4 LDLR and ApoAI

The low density lipoprotein receptor (LDLR) is a protein with multiple domains, which include extracellular domain consisting of an N-terminal ligand binding domain, followed by the epidermal growth factor (EGF)-precursor homology domain, a single transmembrane segment (TMS) and a small cytoplasmic domain (CD) (Jeon et al., 2001; Russell et al., 1984). The EGF-precursor homology domain contains three EGF-like repeats (A–C) and a β propeller (Jeon et al., 2001; Rudenko and Deisenhofer, 2003; Russell et al., 1984). Because of its ability to bind and release LDL, LDLR plays a crucial role in cholesterol metabolism. At a neutral pH such as in plasma, LDLR binds LDL on the cell surface and internalizes it releasing it in the endosomes at acidic pH. Inside the lysosome the LDL is degraded and LDLR is recycled. Ca^{2+} is needed for binding of LDL by LDLR (Andersen et al., 2003; Fass et al., 1997; Hiesberger et al., 1996). Recently, it was reported (Kwon et al., 2008) that proprotein convertase subtilisin/kexin type 9 (PCSK9) regulated hepatic LDLR by binding to its epidermal growth factor-like repeat A (EGF-A) domain on the cell surface, and led to LDLR degradation.

Apolipoprotein A-I (ApoAI) is the predominant apolipoprotein in high density lipoproteins (HDLs) and represents 80-90% of the total proteins (Eisenberg, 1984). ApoAI plays important role in cholesterol efflux by acting as a receptor for lipid in the initial and obligatory step in HDL particle assembly, which is the addition of lipids to ApoAI by ATP binding cassette transporter A1 (ABCA1) to form different sized pre-β HDLs (Francis, Knopp, and Oram, 1995; Remaley et al., 1997). To undergo further maturation, pre-β HDLs are added along with more lipids by phospholipid transfer protein (PLTP), ABCG1 and/or scavenger receptor class B member 1 (SR-BI) (Gelissen et al., 2006; Oram et al., 2003; Vaughan and Oram, 2006). ApoAI interacts directly with ABCA1(Chambenoit et al., 2001; Fitzgerald et al., 2002; Wang et al., 2001) and the

interaction is reduced with the association of ApoAI with lipids (Denis et al., 2004) but does not depend on membrane phosphatidylcholine or sphingomyelin (Denis et al., 2004). Recent studies (Mulya et al., 2008) showed conformation of the complexes formed by ApoAI and ABCA1 at the initial stage of HDL assembly partially determine the *in vivo* metabolic fate of ApoAI, in which with the increase of pre-β HDL particle size the catabolism of ApoAI was increased in the liver and decreased in the kidney.

6. AIMS OF THE STUDY

To study the regulation of lipid metabolism associated genes by vitamin D in human primary prostate stromal cells and human prostate cancer cells (I, II and III)

To study the interaction of VDR ligand, AR agonist and LXR agonist in human prostate cancer cells (II and III)

To study the level of cholesterol and the expression of lipid metabolism associated genes in VDR knockout mice (IV)

7. MATERIALS AND METHODS

7.1 Reagents (I, II, III and IV)

1α,25$(OH)_2D_3$, 24R,25$(OH)_2D_3$, 25OHD_3, were obtained from Leo Pharmaceuticals (Ballerup, Denmark) and 5α-dihydrotestosterone (DHT) from Merck (Darmstadt, Germany). Cycloheximide, Actinomycin D, Desmesterol (5, 24-cholestadien-3β-ol), 25-hydroxycholesterol (cholest-5-ene-3β, 25-diol), glybenclamide, TO-901317 and RPMI-1640 medium were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Casodex was obtained from AstraZeneca (London, UK). FBS was purchased from Gibco-BRL (Life Technology, Paisley, Scotland). TRIzoL reagent was purchased from Invitrogen (Carlshad, USA). High Capacity DNA Archive Kit and SYBR Green PCR Master Mix Kit were purchased from Applied Biosystems (Forster City, USA). One-Cycle Target Labelling Assay Kit and Human Genome U133 Plus 2.0 GeneChip were purchased from Affymetrix (Affymetrix, Inc. Santa Clara, CA). Photometric CHOD-PAP was purchased from Germany (Ecoline® S+ Cholesterol, DiaSys Diagnostic Systems GmbH, Germany).

7.2 Cell culture and treatment (I, III)

Human prostate cancer cell lines LNCaP clone FGC, DU145 and PC3 (American Type Culture Collection) were cultured in phenol red-plus RPMI-1640 medium, supplemented with 10% FBS or 10% FBS-DCC, 5μg/ml insulin and antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml) at 37°C in a humid atmosphere with 5% CO_2. Human primary prostate stromal cells termed P29SN (Lou et al., 2004) were cultured in phenol red-free DMEM/F12 medium, supplemented with 10% FBS, 3mM L-glutamine, 5μg/ml insulin and antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml) at 37°C in a humid atmosphere with 5% CO_2. For P29SN cells and LNCaP cells that were subjected to DHT treatment, the medium was changed to corresponding 10% FBS-DCC supplemented medium 24h before treatment. Cells were treated with calcitriol or/and other reagents, which were diluted in ethanol or ethanol plus DMSO depending on the solubility of the reagent used**.** For all the treatments, negative control cells were treated with corresponding concentration of diluting agents. Cells were grown to 50% confluence for treatment.

7.3 RNA isolation (I, II, III and IV)

Total cellular RNA was isolated with TRIzoL reagent following the instructions from the manufacturer. The RNA concentration was calculated from absorbance at 260nm in a GeneQuant II (Pharmacia Biotech, USA) and A280/A260 was measured to verify the purity of the RNA. The values of A280/A260 from all the RNA samples were in between 1.8 and 2.1. Randomly selected RNA samples were subjected to denaturing-gel electrophoresis. RNA was stored at -70 ºC. All the RNA used was thawed only once.

7.4 Affymetrix c-DNA microarray (I)

7.4.1 cRNA preparation and cDNA microarray hybridization

7µg total RNA was used as template to synthesize double-stranded cDNA. The cDNA was used as template to generate biotynylated cRNA by an *in vitro* transcription reaction. 20µg of biotynylated cRNA was fragmented and added to the U133 Plus 2.0 chip. The hybridization was carried out at 45^{o}C for 20 hours with a rotation at 60 rpm in the Affymetrix Hybridization oven. Subsequently, the arrays were washed and stained with a streptavidin-conjugated fluorescent stain followed by antibody amplification on the Affymetrix Fluidics Station 400. The chips were scanned and images were processed using Affymetrix GeneChip Operating Software Server 1.0 (GCOS Server) (Affymetrix, Santa Clara, CA) and raw data was normalised and analysed using GENESPRING software (Version 7.2, Silicon Genetics, Redwood City, CA).

7.4.2 Data analysis

The raw data from the array scans was normalized and analyzed with GeneSpring GX 7.3.1 Expression Analysis software (Agilent Technologies). Briefly, the data was transformed where values < 0.01 were converted to 0.01 to enable more efficient analysis of log-transformed data. To control chip-wide variation in intensity values, each chip was normalized to the 50th percentile of all measurements (per-chip normalization). As per gene normalization the treated samples were normalized against the median of the control samples. The measurement for individual gene in those vitamin D metabolites treated samples was divided by the median of the measurements of the corresponding control samples. In all of these 3 conditions the present call was demanded only to respective conditions. In the hierarchical two-dimensional clustering algorithm genes are represented by each row and experimental samples represented on each column. We used Pearson Correlation as similarity measure and unsupervised average linkage hierarchical clustering algorithm. The program was asked to merge similar branches with separation ratio of one and minimum distance of 0.001 and to calculate confidence levels (Bootstrapping) with 100 data sets. Venn diagrams were produced separately to up and down regulated genes in each three conditions.

7.5 *In vivo* experiments (IV)

7.5.1 Mouse breeding, housing and feeding

VDR-KO mice 129S1 were produced from the line initially generated in the University of Tokyo (Yoshizawa et al., 1997) and have been studied in our laboratory (Kalueff et al., 2006; Keisala et al., 2007; Minasyan et al., 2007). NMRI mice were purchased from Harlan, Nederland. They originate from Swiss mice in the US brought from Lausanne, Switzerland, in 1926 by Clara Lynch. In 1937 the mice came from Lynch to Poiley and were inbred by Poiley known as NIH/PI. At F51 they went to US Naval Medical Research Institute and thus known as HsdWin:NMRI (NMRI in the text). In 1955, the mice went to Bundes-Forschungsanstalt für Viruskrankheiten and in 1958, to Central Institute for Laboratory Breeding, Hannover. In 1981, they came from Central Institute for Laboratory Breeding, Hannover to Winkelmann, now Harlan Winkelmann. In 1998,

they came from Harlan Winkelmann to Harlan Nederland (www.harlaneurope.com), where we bought the mice for the present study. All the mice were housed in the University of Tampere Laboratory Animal facility with a 12:12-light:dark cycle. Mice were genotyped before experimental studies. The VDR-KO mice were fed with special diet containing 2% Ca, 1.25% P and 20% lactose (Lactamin AB, Sweden) and WT mice with normal diet containing 0.9% Ca, 0.7% P and 20% lactose (Lactamin AB, Sweden). In the studies with NMRI mice, WT mice were switched from normal foods to special foods 3 weeks before sample collection.

7.5.2 Serum sample preparation and tissue sample collection

Mice were sacrificed by carbon dioxide and blood was immediately taken by heart puncture. Blood was allowed to clot, followed by centrifugation at 3000rpm for 10min. The serum was stored at -70ºC for further analysis. Small pieces of liver tissues were taken, dropped into RNAlater (Ambion), and stored at -20ºC for later RNA isolation.

7.5.3 Measurement of total cholesterol and HDL-C

Total cholesterol and HDL-C concentrations were measured using a photometric CHOD-PAP (Ecoline® S+ Cholesterol, DiaSys Diagnostic Systems GmbH, Germany). HDL-C was determined from the clear supernatant after precipitation of serum apoB-containing lipoproteins with 10% polyethylene glycol (final concentration).

7.6 Quantitative real-time PCR (I, II, III, IV)

7.6.1 Reverse transcription

3-8µg total cellular RNA was used to synthesize cDNA by using High Capacity Archive Kit (Applied Biosystems, USA) in a final volume of 100µl. 0.5-2µl of un-diluted cDNA reactions or 4-8µl of 1:10 diluted cDNA reactions were used as input for each of the real-time quantitative PCR reactions by using SYBR Green PCR Master Mix kit (Applied Biosystems, USA). The reactions were carried out in GeneAmp PCR System 2400 (Perkin Emer, Oak Brook, IL, USA). The program for the reactions were: transcription activation at 25ºC for 10min followed by reverse transcription at 37ºC for 2h and ended with enzyme inactivation at 95ºC for 5min.

7.6.2 Primer design

Primers were designed using Primer Express v2.0 software (Perkin-Elmer Applied Biosystems, Foster City, CA). BLASTn searches were performed to ensure that the primers were gene specific and intron-spanning. CH25H gene spans one exon, real-time PCR using un-reverse-transcribed RNA as template was performed to ensure that the experimental results were not compromised through genomic DNA contamination. The primers used in this thesis are in Table 1.

Table1. Primers for quantitative real-time PCR analysis ("m" is the abbreviation for "mouse")

Gene	Nucleotide sequence	GeneBank accession
CH25H	Forward: 5′-TCCTGTTCTGCCTGCTACTCTTC-3′ Reverse: 5′-GGTACAGCCAGGGCACCTT-3′	NM_003956
CYP27A1	Forward: 5′-GAGTGGACACGACATCCAACAC-3′ Reverse: 5′-CTCCTGGATCTCAGGGTCCTT-3′	M62401
RPLP0	Forward: 5′-AATCTCCAGGGGCACCATT-3′ Reverse: 5′-CGCTGGCTCCCACTTTGT-3′	NM_001002
HSD17B2	Forward: 5′-GGCCATGCTTTGTGCAAGT-3′ Reverse: 5′-TCATTCAAAACTCCGGCAAAT-3′	BC059170
HSD17B4	Forward: 5′-TTGGGCCGAGCCTATGC-3′ Reverse: 5′-CCCCTCCCAAATCATTCACA-3′	NM_000414
HSD17B5	Forward: 5′-GGGATCTCAACGAGACAAACG-3′ Reverse: 5′-AAAGGACTGGGTCCTCCAAGA-3′	NM_003739
PBGD	Forward: 5′-CACACACAGCCTACTTTCCAA-3′ Reverse: 5′-TTTCTTCCGCCGTTGCA-3′	X04808
GAPDH	Forward: 5′-CCACATCGCTCAGACACCAT-3′ Reverse: 5′-ACCAGGCGCCCAATACG-3′	NM_002046
ABCA1	Forward: 5′-GAGCACCATCCGGCAGAA-3′ Reverse: 5′-CTCCGCCTTCACGTGCTT-3′	NM_005502
ABCG1	Forward: 5′-GCTGCTGCCGCATCTCA-3′ Reverse: 5′-TTCCCTTCTGCCTTCATCCTT-3′	NM_207630
LXRα	Forward: 5′-CATGCCTACGTCTCCATCCA-3′ Reverse: 5′-CGGAGGCTCACCAGTTTCA-3′	HSU22662
LXRβ	Forward: 5′-GATGTCCCAGGCACTGATGA-3′ Reverse: 5′-CTGGTTCCTCTTCGGGATCTG-3′	HSU07132
VDR	Forward: 5′-CCTTCACCATGGACGACATG-3′ Reverse: 5′-CGGCTTTGGTCACGTCACT-3′	NM_000376
CYP24	Forward: 5′-GCCCAGCCGGGAACTC-3′ Reverse: 5′-AAATACCACCATCTGAGGCGTATT-3′	NM_000782
mABCA1	Forward: 5′-CAACCCCTGCTTCCGTTATC-3′ Reverse: 5′-GACCTTGTGCATGTCCTTAATGC-3′	NM_013454
mApoAI	Forward: 5′-CTCCTCCTTGGGCCAACA-3′ Reverse: 5′-TGACTAACGGTTGAACCCAGAGT-3′	NM_009692
mLDLR	Forward: 5′-TGTGAAAATGACTCAGACGAACAA-3′ Reverse: 5′-GGAGATGCACTTGCCATCCT-3′	NM_010700
mLXRβ	Forward: 5′-GATCCTCCTCCAGGCTCTGAA-3′ Reverse: 5′-TGCGCTCAGGCTCATCCT-3′	NM_009473
mSREBP2	Forward: 5′-GTGCGCTCTCGTTTTACTGAAGT-3′ Reverse: 5′-GTATAGAAGACGGCCTTCACCAA-3′	NM_033218
mβACTIN	Forward: 5′-GCTTCTTTGCAGCTCCTTCGT-3′ Reverse: 5′-CCAGCGCAGCGATATCG-3′	NM_007393

7.6.3 PCR amplification

Real-time PCR was performed with the ABI PRISM 7000 Detection System (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Applied Biosystems). The programs for the amplification were as following: activation of polymerase at 95ºC for 10min, followed by 45 cycles of denaturation at 95ºC for 15sec and annealing/extension at 60ºC for 1min. The analysis of dissociation curves was always performed after 45 cycles.

7.7 RNA stability assay (I)

Cells were pre-treated with 0.1% ethanol or 10nM 1α,25$(OH)_2D_3$ for 21h followed by incubation in the presence of actinomycin D for 0 hours, 2 hours, 4 hours and 8 hours. Total RNA from each time point was isolated and quantified by real-time PCR. The relative expression level of mRNA was then analysed by GraphPad Prism software (GraphPad Prism version 4.03 for Windows, www.graphpad.com), which gave the best-fit curves for subject and control mRNA degradation and the half-lives of corresponding mRNA.

7.8 Cell growth assay (I and III)

Cells were seeded in the wells of plates and allowed to sit for 24h followed by treatment with hormones and other reagents for 0 days, and other time periods. Three experiments for each treatment were repeated. In article I, 1400 cells in 100µl of medium (P^9, P29SN) were seeded in each well of 96-well plate (Nunc, Denmark). In article III, $5.5*10^4$ cells in 2ml of medium (P^{11}, LNCaP) were seeded in each well of CellBIND 6-well plate (Sigma-Aldrich). Cell density was analysed with crystal violet staining. Briefly, cells were fixed by addition of glutaraldehyde to the cell culture with a final concentration of 1% and shaking at 200rpm for 15min, then washed with tap water and air-dried over night. 0.1% crystal violet solution was added to stain the fixed cells for 20min with shaking at 200rpm. Excess dye was removed by extensive washing with tap water. The plates were air-dried over night. 10% acetic acid was used to withdraw cell-bound dye. The optical density of extracted dye was measured in 96-well plates at 590nm by Microplate Reader (Wallac, victor 1420 multilabel counter, Turku, Finland). The final data was presented as absorbance or percentage absorbance of negative control cells for each corresponding treatment period as indicated.

7.9 Statistics (I, II, III and IV)

For articles I, II and III, the data was expressed as the mean values ± standard deviations. Significance was assessed by using the Independent paired t-Test. For article IV, the data was presented as the mean values ± standard errors and the significance was assessed by using the Mann-Whitney *U* test. In all four articles, $*p \leq 0.05$ was considered significant, $**p < 0.001$ as highly significant and $p > 0.05$ as not significant (NS).

8. RESULTS

8.1 The regulation of CH25H by vitamin D (I)

8.1.1 CH25H mRNA expression

8.1.1.1 cDNA microarray analysis of vitamin D regulated lipid metabolism associated genes

A survey of vitamin D regulated genes in human primary prostate stromal cells was performed by using Human Genome U133 Plus 2.0 Array chip representing over 47,000 transcripts, which include 38,500 well-characterized human genes. The results revealed that cholesterol 25-hydroxylase (CH25H) was not regulated by 24,25$(OH)_2D_3$ but up-regulated by 1α,25$(OH)_2D_3$ and 25OHD_3 by 3.17 and 1.74 fold, respectively. *ch25h* is a lipid metabolism associated gene and thus was selected for further investigation.

8.1.1.2 Regulation of CH25H expression by vitamin D_3 in human primary prostate stromal cells

To further confirm the above observation, quantitative real-time PCR (QRT-PCR) was performed. It was shown that 10nM 1α,25$(OH)_2D_3$ treatment for 24h increased CH25H mRNA expression levels by 3.4 ± 0.65 (P = 1.7E - 05) fold, 0.5nM 1α,25$(OH)_2D_3$ and 25nM 24,25$(OH)_2D_3$ had no significant effects on CH25H expression. 10nM 1α,25$(OH)_2D_3$ and 500nM 25OHD_3 reached maximum CH25H mRNA induction at 12h and remained high until 48h. At 24h 500nM treatment, 25OHD_3 increased CH25H expression by 2.6 ± 0.51 (P = 7.8E - 05) fold, and no change was observed at 50nM, 100nM or 250nM.

8.1.2 Effects of 1α,25$(OH)_2D_3$ on CH25H mRNA stability

QRT-PCR showed that cycloheximide had no significant effect on calcitriol mediated CH25H mRNA induction, suggesting the regulation of CH25H does not need de novo protein synthesis. To study whether the increase of CH25H mRNA level is due to the increased stability of the corresponding mRNA, actinomycin D (act D) was used. Initial data indicated that both basal and calcitriol mediated up-regulation of CH25H mRNA was blocked by act D treatment, indicating that calcitriol mediated up-regulation of CH25H is transcription dependent. Next, cells pre-stimulated with ethanol or 1α,25$(OH)_2D_3$ for 21h had act D added at 0h, 2h, 4h, 6h and 8h time periods. The mRNA expression levels were analysed and plotted against each treated time point. The slops of corresponding best-fit curves representing the mean half-life of CH25H mRNA were -10.20 ± 3.998 (r^2 = 0.7650) for ethanol and -10.36 ± 4.692 (r^2 = 0.7092) for 1α,25$(OH)_2D_3$. The difference between the two slopes and hence the half-lives was not significant (P = 0.9806).

8.1.3 Effects of CH25H inhibitor and 25-hydroxycholesterol on P29SN cell proliferation

To determine whether the induction of CH25H by vitamin D contributes to its mediation of cell proliferation, 25-hydroxycholesterol and CH25H inhibitor desmosterol, were used in a cell growth study. 25-hydroxycholesterol inhibited the proliferation of P29SN cells. Desmosterol significantly increased cell proliferation by 40% at Day 9 (P = 0.02). The effect of desmosterol on calcitriol-mediated antiproliferation was under the limit of detection.

8.2 Cross-talk between VDR ligand and LXR agonist in mutual regulation of CYP24 and ABCA1 (III)

8.2.1 Effects of calcitriol on LXR signaling related gene expression

The product of CH25H is 25-hydroxycholesterol, which is one of the natural occurring ligands of LXR. ABCA1 is a primary target gene of LXR (Costet et al., 2000; Repa et al., 2000), thus we were interested to study whether vitamin D has any effects on ABCA1 expression. In P29SN cells, we failed to detect any regulation. However, in prostate cancer LNCaP cells, the expression of ABCA1 mRNA was dose and time dependently inhibited by $1\alpha,25(OH)_2D_3$. At 24h treatment, 1nM $1\alpha,25(OH)_2D_3$ decreased ABCA1 mRNA expression by 47% (P = 0.005) and 10nM decreased ABCA1 mRNA expression by 75% (P = 8.8E - 07). Furthermore, 10nM calcitriol decreased an LXR agonist, TO-901317, induced ABCA1 expression by 47% (P = 0.017). On the other hand, $1\alpha,25(OH)_2D_3$ had no effects on both LXRα and LXRβ expression, and it had no effect on either basal nor TO-901317 induced ABCG1 expression, which is another reported LXR direct target gene.

8.2.2 Effects of TO-901317 on CYP24 and VDR expression

We also studied whether the LXR agonist has effects on VDR mediated signaling. Our data showed that TO-901317 inhibited the VDR mRNA expression only slightly but significantly. Calcitriol addition had no statistically significant effects on this inhibition. TO-901317 increased basal CYP24 mRNA expression 8 ± 1.7 fold (P = 0.03). Furthermore, it enhanced calcitriol-mediated induction of CYP24 expression 10 ± 1 fold (P = 2E - 04).

8.2.3 The role of calcitriol mediated ABCA1 inhibition in LNCaP cell proliferation

To study whether the inhibition of ABCA1 by calcitriol rendered any physiological effect regarding LNCaP growth, and to compare the effects of calcitriol and DHT on LNCaP cell proliferation when they were individually co-treated with glybenclamide (ABCA1 inhibitor) or TO-901317, a cell growth assay was performed. At day 7, Calcitriol and DHT each by itself decreased LNCaP cell growth by 61% (P = 8.5E - 07) and 69% (P = 5.4E - 06), respectively. When calcitriol and DHT was each co-treated with glybenclamide, cell growth was decreased by 29% (P = 0.01) and 39% (P = 0.01) compared to their own treatment respectively. However, when co-treated with TO-

901317, calcitriol showed no significant difference whereas DHT showed a significant decrease of cell proliferation by 32% (P = 0.03), when compared with each hormone treatment alone respectively. TO-901317 and glybenclamide individually decreased LNCaP cell growth. Their co-treatment further decreased cell proliferation, which remained unchanged whether when calcitriol or DHT was added.

8.3 Interactions between androgen, VDR ligand and LXR agonist signaling (I, II and III)

8.3.1 Effects of DHT on CH25H mRNA expression

DHT decreased CH25H mRNA expression only slightly but significantly and it had no effect on calcitriol-induced CH25H mRNA expression.

8.3.2 Androgen-dependency of ABCA1 expression

DHT decreased ABCA1 expression, which was in line with an earlier study (Fukuchi et al., 2004a). In medium containing normal serum, the androgen receptor antagonist, bicalutamid, increased significantly ABCA1 mRNA expression by 1.6 ± 0.3 fold (P = 0.02), and the addition of calcitriol reverted it to the level with calcitriol treatment alone. Furthermore, in low-androgen-serum (dextran-charcoal-stripped serum) containing medium, 10nM calcitriol still significantly decreased ABCA1 expression by 64% (P = 4.8E - 06). This suggests that the inhibition of ABCA1 by calcitriol is most probably androgen-independent.

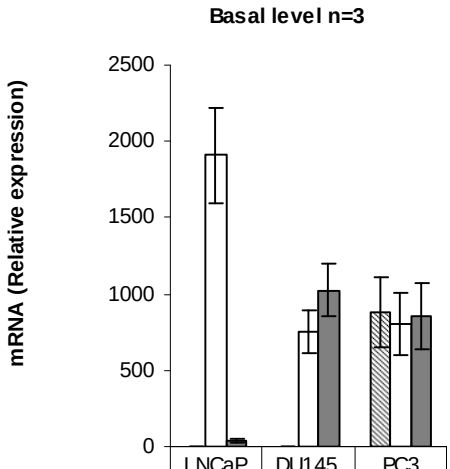


Figure 1. Basal expression of HSD17B2, 4 and 5 in LNCaP, DU145 and PC3

LNCaP, DU145 and PC3 cells cultured in medium containing normal serum were lysed for total RNA isolation. The relative mRNA of HSD17B2, 4 and 5 were measured by QRT-PCR. The data represented is the average of the results from three independent experiments.

8.3.3 Effects of VDR ligands, LXR agonists and androgens on the expression of HSD17B2, HSD17B4 and HSD17B5

8.3.3.1 Basal expression of HSD17B2, HSD17B4 and HSD17B5

The basal expression level of 17beta-hydroxysteroid dehydrogenase type 2, type 4 and type 5 mRNA in human prostate cancer cells, LNCaP, DU145 and PC3, was determined by quantitative real-time PCR (Figure 1). The results demonstrated that in PC3 cells, all three enzymes had similar mRNA expression levels. However, in LNCaP cells, they differ clearly with high expression level of type 4. In DU145 cells, type 4 and type 5 had similar mRNA levels and the expression level of type 2 mRNA was very low.

8.3.3.2 Effects of calcitriol and an LXR agonist (TO-901317) on HSD17B2 expression

Calcitriol up-regulated HSD17B2 mRNA expression time (Fig 2B) and dose (Fig 2A) dependently in both LNCaP and PC3 cells. Our results showed that in both cell lines, the highest HSD17B2 mRNA expression occurred at 100nM of calcitriol and/or 48h of 10nM calcitriol treatments, which were the highest dose and/or the longest time period with fixed concentration of calcitriol treatment in our study, indicating a further potential of increase with bigger dosage or prolonged treatments. This seems more obvious in LNCaP cells as shown in Figure 2A and 2B. In human primary prostate stromal cells, 10nM calcitriol showed no effect on HSD17B2 expression at 30min, 4h, 12h, 24h or 48h.

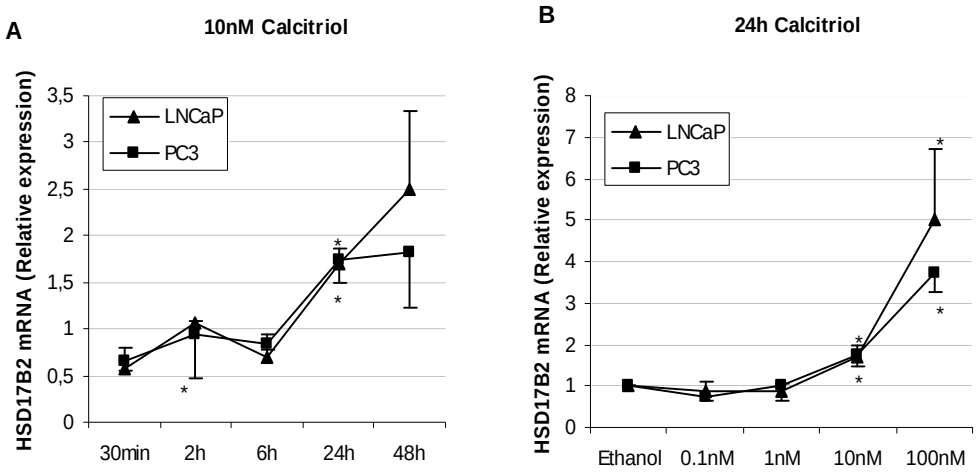


Figure 2. Effects of calcitriol on HSD17B2 in LNCaP and PC3 cells. Cells were treated with different doses of calcitriol for 24h (A, *p < 0.05) or 10nM of it for various time period (B, *p < 0.05). Relative expression of HSD17B2 mRNA was analyzed with QRT-PCR. For clarity, only plus Y-error bars were shown for LNCaP cells and minus ones for PC3 cells.

10μM TO-901317 treatment of LNCaP and PC3 cells for 24h significantly decreased HSD17B2 mRNA expression by 91% (P = 2.7E - 07) and 30% (P = 0.03), respectively. Furthermore, it inhibited calcitriol-induced expression of HSD17B2 mRNA by 95% (P = 0.0004).

8.3.3.3 Effects of calcitriol and TO-901317 on HSD17B4 and HSD17B5 mRNA expression

In LNCaP cells, 10µM TO-901317 at 24h up-regulated HSD17B5 2.0 ± 0.16 fold (P = 0.01) but had no effect on HSD17B4 expression. The induction of HSD17B5 by TO-901317 remained unchanged when co-added with calcitriol. 10nM calcitriol increased respectively the expression of HSD17B4 and HSD17B5 by1.6 ± 0.16 (P = 0.03) and 1.7 ± 0.12 (P = 0.02) fold at 48h but had no effects either at 30min, 4h, 12h or 24h. However, when LNCaP cells were cultured in DCC-serum containing medium, the effects of calcitriol on both HSD17B4 and 5 were lost. In human primary prostate stromal cells, calcitriol showed no effect on HSD17B4 expression at 30min, 4h, 12h, 24h or 48h. As to HSD17B5, it showed 1.4 ± 0.2 fold (P = 0.04) induction at 12h but not at other time points.

8.3.3.4 Effects of DHT on HSD17B2, HSD17B4 and HSD17B5 mRNA expression

In LNCaP cells cultured in DCC-FBS supplemented medium, 10nM DHT up-regulated HSD17B2 mRNA 20000 ± 1700 fold at 24h (P = 0.003), which was not affected by calcitriol and which remained similar when an alternative endogenous control gene, PBGD, was used for normalization during QRT-PCR analysis. In the same experimental condition, DHT significantly up-regulated HSD17B4 mRNA expression 1.6 ± 0.06 (P = 5.6E - 05) fold, but it significantly down-regulated HSD17B5 mRNA expression by 70% (P = 5.4E - 07). Calcitriol showed no effects on DHT mediated HSD17B4 and HSD17B5 expression.

8.4 Studies with VDR knockout mice (IV)

8.4.1 Strain 129S1

8.4.1.1 Total cholesterol and HDL-C in wild-type and VDR knockout mice

To study whether the vitamin D receptor had an effect on serum total cholesterol and

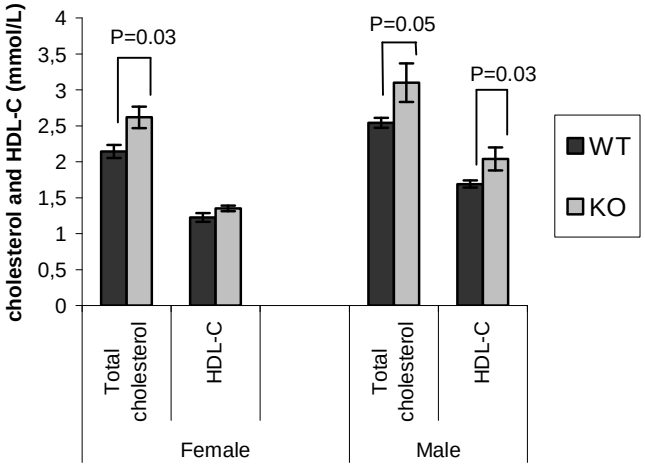
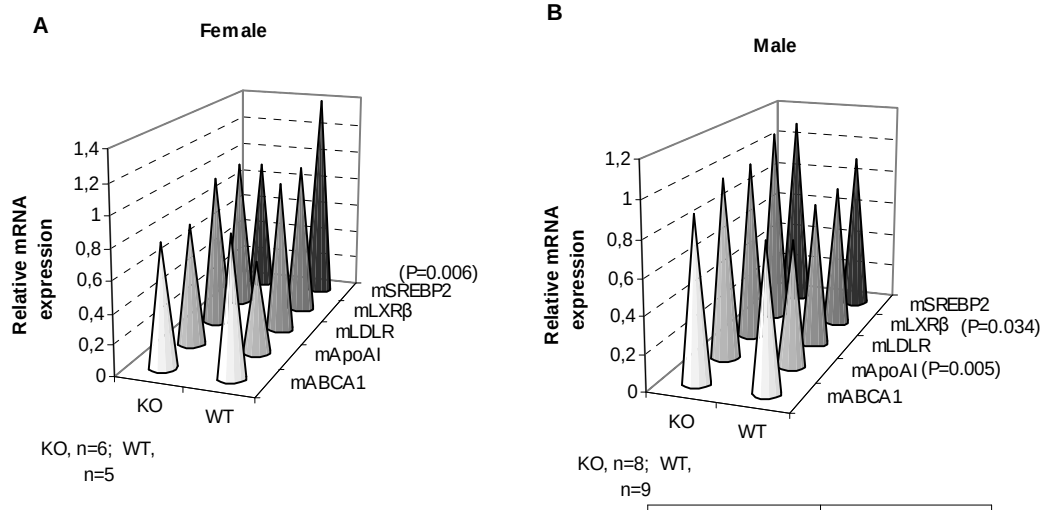


Figure 3. Cholesterol and HDL-C level in wild-type and VDR knockout mice. The data was analyzed by the Mann-Whitney *U* test and represented as mean value ± standard error. The unit used in the table is mmol/L (SI Unit). To convert values from SI units to conventional units, divide by the conversion factor. For cholesterol and HDL-C, the convert factor is 0.0259. The numbers of the mice studied were: female KO n = 6, female WT n = 5; male KO n = 8, male WT n = 9.

HDL-C, blood was taken from VDR-KO and WT mice, and serum was separated simultaneously. The mean total cholesterol concentration was significantly higher in female KO mice in comparison with that of female WT mice (Figure 3). However, there was no statistically significant difference between the two types of mice for HDL-C. In male mice, both total cholesterol and HDL-C concentrations were significantly lower in WT mice compared to those in KO mice (Figure 3).

8.4.1.2 ABCA1, ApoAI, LDLR, LXRβ and SREBP2 mRNA expression from liver tissue

Our QRT-PCR study of lipid homeostasis related genes in the liver tissues of WT and VDR-KO mice showed that the lack of functional VDR had different effects on female and male mice. Female VDR-KO mice (Figure 4A) showed a significantly lower SREBP2 mRNA level in the liver than that of wild-type mice. In males (Figure 4B), the mutant mice livers significantly express higher levels of ApoAI and LXRβ mRNA.



	KO	WT
mABCA1	0,8	0,9
mApoAI	0,8	0,6
mLDLR	1	1
mLXRβ	1	1
mSREBP2	0,9	1,4

	KO	WT
mABCA1	0,9	0,8
mApoAI	1	0,7
mLDLR	1	0,8
mLXRβ	1,1	0,8
mSREBP2	1,1	0,9

Figure 4. The liver mRNA expression of ABCA1, ApoAI, LDLR, LXRβ and SREBP2 in wild-type and VDR-KO mice. mRNA from female (A) and male (B) was analyzed with QRT-PCR. Mouse β-ACTIN was used as a control gene for normalization during relative mRNA quantification.

8.4.2 Strain NMRI

Since 129S1 VDR-KO mice were fed with special food for the balance of calcium, the results above might be affected by the difference of the food, we fed NMRI WT mice with the same food as KO mice for 3 weeks before blood sample collection. The result

showed no significant difference for total cholesterol and HDL-C between WT and KO mice, but a clear sexual difference was found.

9. DISCUSSION

9.1 The regulation of CH25H, ABCA1 by vitamin D (I and III)

9.1.1 Effects of vitamin D on CH25H in human primary stromal cells

In the search of vitamin D regulated lipid metabolism associated genes in human primary stromal cells, we performed cDNA microarray analysis. Less than five lipid metabolism associated genes were identified, among which CH25H was the most prominent one and it appeared to be regulated by both calcitriol and calcidiol. Reported lipid metabolism related genes which are regulated by vitamin D include human PPARδ (Dunlop et al., 2005), mice Insig-2 (Lee et al., 2005) and human ApoAI (Wehmeier et al., 2005). In prostate cancer LNCaP cells, Qiao et al. (Qiao et al., 2003) reported that calcitriol inhibited fatty acid synthase expression via stimulating the expression of long-chain fatty-acid-CoA ligase 3 (Qiao and Tuohimaa, 2004a; Qiao and Tuohimaa, 2004b). These studies were carried out in breast, prostate and liver cancer cells, which are mostly epithelial cells. In this study, stromal cells were used, and for the first time, we found that *ch25h* was regulated by vitamin D.

The regulation seemed to be direct because protein synthesis was not needed for the regulation of CH25H by vitamin D_3, and CH25H mRNA stability was not affected by calcitriol. This suggests that the promoter of *ch25h* might harbour VDRE(s) for the binding of proteins for transcription. This implication is supported by the presence of a DR3-type vitamin D responsive element (VDRE), caactcagttcacgtgtgtcatcac, at position −203 to −179 upstream of the translation initiation site in the promoter of *ch25h* gene, predicted by the GENOMATIX Software MatInspector (http://www.genomatix.de). Whether this is active or not need further study to verify.

To study whether the regulation of CH25H expression by vitamin D render any physiological effects, cell growth assays were performed by employing 25-hydroxycholesterol and desmosterol, which are the product and the inhibitor of CH25H respectively. 25-hydroxycholesterol inhibited the synthesis of cholesterol (Adams et al., 2004; Brown and Goldstein, 1999). Cholesterol synthesis is the prerequisite for cell proliferation, given the fact that the membranes of the cells are constructed mainly with cholesterol. Previous studies clearly demonstrated that calcitriol was involved in the control of cell proliferation. Therefore the question was whether CH25H induced by calcitriol contributed to the antiproliferative action. First, 25-hydroxylcholesterol significantly decreased prostate cell number. Secondly, desmosterol promoted basal cell proliferation significantly, implying that the inhibition of basal level of cholesterol 25-hydroxylase promoted the growth of the cells. The effect of desmosterol on calcitriol exerted growth inhibition was not found. This might be due to the dosage of desmosterol used could inhibit the basal level of the cholesterol 25-hydroxylase activity but was not high enough to inhibit calcitriol up-regulated enzyme activity. Thus, it would be good to include enzyme activity analysis in future studies. Meanwhile, the P29SN cells were growth inhibited by 25-hydroxylcholesterol in a concentration dependent manner. The examination of 25-hydroxylcholesterol concentration levels after vitamin D treatment

will be also meaningful. The regulation of CH25H by calcitriol has been difficult to investigate in prostate cancer LNCaP cells, due to the very low level of its mRNA expression.

Nevertheless, this provides the first evidence that vitamin D_3 may directly up-regulate cholesterol 25-hydroxylase. Since 25-hydroxycholesterol inhibited prostate cell growth and cholesterol 25-hydroxylase inhibitor, desmosterol, enhanced basal cell proliferation, we propose that vitamin D_3-mediated antiproliferation may be partially due to the upregulation of cholesterol 25-hydroxylase.

Serum cholesterol level seems to be inversely related to vitamin D_3 levels (Grimes, Hindle, and Dyer, 1996). However, the mechanism behind this phenomenon is not clear. 25-hydroxycholesterol is a potent inhibitor of cholesterol synthesis, and therefore, the up-regulation of CH25H by vitamin D_3 may provide at least partially an explanation of how vitamin D_3 can control serum cholesterol level. For further exploration, we used human hepatocellular liver carcinoma cells, HepG2, to study the effect of calcitriol on CH25H, given that the liver plays a pivotal role in cholesterol metabolism. However, unpublished data showed that 10nM of calcitriol at 24 hour treatment had no effect on the mRNA expression of CH25H in HepG2. This is possibly due to the low response, if any, of the cell to vitamin D treatment. QRT-PCR data showed a 2.9 ± 0.6 ($P = 0.005$) fold induction of CYP24 by 10nM calcitriol at 24 hours treatment in HepG2. This is much lower than 3100 ± 290 ($P = 0.007$) fold in P29SN cells, where calcitriol showed a 3.4 ± 0.65 ($P = 1.7E - 05$) fold induction of CH25H. Another cell line, HaCat (human keratinocyte cell line), has been reported to be growth inhibited by vitamin D via down-regulation of CXCR2 expression (Tang et al., 2003) and has been used as a model system for vitamin D_3 metabolism in human skin (Lehmann, 1997), did not show any changes of CH25H mRNA expression after 10nM of calcitriol treatment for 24 hours. Further investigations with cells which show vitamin D's induction of CH25H are needed, especially for cells involved in lipid metabolism.

9.1.2 The effects of vitamin D on ABCA1 in human prostate cancer cells

The product of CH25H is 25-hydroxycholesterol, which is one of the natural ligand for LXR. Upon the binding of its ligand, LXR acts as a transcription factor to stimulate the expression of target genes such as ABCA1 and ABCG1. Thus, we were interested in studying whether vitamin D had a stimulation effect on ABCA1. In the same cells where we found vitamin D up-regulated CH25H, we did not see any effects of vitamin D on ABCA1. Moreover, in prostate cancer LNCaP cells, our data showed that calcitriol inhibited rather than stimulated the expression of ABCA1. The regulation of ABCA1 by vitamin D in LNCaP cells hasn't been reported before. On the other hand, the expression level of CH25H has been too low to study the full effect exerted by vitamin D in LNCaP cells. Thus, the pathway "CH25H—25-hydroxycholesterol—ABCA1" seemed to be broken. The effect of vitamin D on ABCA1 expression has also been examined in HaCat, HepG2 and mouse macrophage Raw 264.7 cells and the results showed no changes after 10nM of calcitriol treatment for 24 hours. In addition, this thesis investigated vitamin D

and lipid metabolism and prostate cancer. Thus, the study has been mainly conducted in LNCaP cells.

In LNCaP cells, the inhibition of ABCA1 mRNA by calcitriol wasn't observed at 2h, and at 6h only minor effects although statistically significant, suggesting an indirect effect on transcription of the ABCA1 mRNA. Indeed, protein synthesis inhibitor, cycloheximide, blocked calcitriol mediated decrease of ABCA1 mRNA. However, it failed to block LXR agonist, TO-901317, mediated induction of ABCA1 and ABCG1, suggesting ABCA1 and ABCG1 are directly regulated by LXR, which is in line with earlier studies (Costet et al., 2000; Karten et al., 2006).

Previously, it has been reported that RNA interference specific knock-down of ABCA1 expression resulted in an increase of LNCaP proliferation (Fukuchi et al., 2004a). They also demonstrated that DHT inhibited the expression of ABCA1 and this inhibition was involved in DHT mediated LNCaP proliferation (Fukuchi et al., 2004a). Our findings also indicated that calcitriol inhibits the expression of ABCA1. Thus, we were interested to test whether the inhibition of ABCA1 by calcitriol had effect on cell proliferation, and DHT was used as a parallel in our cell growth assay. Out of anticipation, we did not observe an increase of cell proliferation after glybenclamide treatment, given that knock-down of ABCA1 increased LNCaP proliferation (Fukuchi et al., 2004a). This might be because glybenclamide can inhibit a broad range of ABC transporters, including ABCA1 and cystic fibrosis transmembrane conductance regulator (Haskó et al., 2002). Thus the final output of specific blocking of ABCA1 by glybenclamide was not seen here. This explains why co-treatment of glybenclamide and TO-901317 did not give a cell growth level which was in between of the level from each reagent treatment alone, but it was even further decreased compared to either of the reagent treatment. Thus, here we could not draw any conclusions by comparing calcitriol treatment and calcitriol plus glybenclamide treatment. However, when each of calcitriol and DHT was co-treated with glybenclamide, cell-growth was significantly decreased compared to their own treatment respectively. This suggests that the regulation of LNCaP cell proliferation by calcitriol may involve inhibition of ABCA1, which has some similarity to DHT, given that inhibition of ABCA1 by DHT is involved in its regulation of LNCaP proliferation (Fukuchi et al., 2004a). However, when co-treated with TO-901317, calcitriol showed no significant difference whereas DHT showed a significant decrease of cell proliferation when compared with each hormone treatment alone suggesting that DHT is more involved in LXR agonist related cell growth regulation than calcitriol. It is worth noting that Fukuchi et al. used a cell line which was derived from LNCaP, namely LNCaP 104-S (Kokontis et al., 1994; Kokontis, Hay, and Liao, 1998), but we used LNCaP cells. In their experiments, androgen, R1881, increased 104-S cell proliferation, whereas we observed a decrease of LNCaP proliferation by DHT. In our lab, using the same LNCaP cells, other researcher also found an inhibition of cell growth by DHT (Lou and Tuohimaa, 2006). At different concentrations, androgens can either induce or repress cell proliferation (Kokontis et al., 1994).

9.2 Interaction between androgen, VDR ligand and LXR agonist signaling (I, II, III and IV)

Previous studies suggest that the actions of vitamin D on prostate cells exist in both androgen-dependent and -independent ways (Zhao et al., 1997; Zhao et al., 2000). In the present study, we demonstrated that in the prostate stromal cells cultured in DCC-FBS (androgen depleted) medium, calcitriol still up-regulated CH25H, suggesting this regulation was androgen-independent. This was confirmed by the fact that 5α-dihydrotestosterone (DHT) addition had no significant effect on vitamin D regulation of CH25H mRNA. In LNCaP cells, we found for the first time that calcitriol inhibited ABCA1, which has been reported to be inhibited by androgen (Fukuchi et al., 2004a). Our study showed in normal medium, AR antagonist, bicalutamide, increased the level of ABCA1, a primary LXR target gene, and calcitriol acts as an antagonist of bicalutamide. Furthermore, calcitriol significantly inhibited ABCA1 in DCC-FBS containing medium. This suggests that the inhibition of ABCA1 by calcitriol is most probably androgen-independent. In a search for vitamin D regulated lipid metabolism associated genes in NCBI, we found HSD17B2 was up-regulated by vitamin D in a microarray study (Wang et al., 2005b), our QRT-PCR confirmed this. HSD17B2 is an enzyme responsible for androgen inactivation. Therefore we studied the interaction between the ligands of three nuclear receptors, namely VDR, AR and LXR, mediated signaling, with the mRNA expression level changes of corresponding receptor related genes as an indicator.

The up-regulation of HSD17B2 by calcitriol occurred in normal serum but not in DCC-serum supplemented medium, indicating that this regulation might be androgen dependent. However, in AR-negative PC3 cells, calcitriol induced HSD17B2. This implies that the functional AR might be dispensable for the induction of HSD17B2 by calcitriol, but the presence of androgen in the medium is necessary. Further studies by examination of the effects of calcitriol on HSD17B2 mRNA level changes in PC3 cells cultured in DCC-serum medium can help to test this hypothesis. There is no study concerning the effect of androgen on the expression of HSD17B2 although there are studies indicating that changes of HSD17B2 activity result in changes of androgen levels (Castagnetta et al., 1997; Härkönen et al., 2003; Luu-The, 2001). Our present study showed that androgen up-regulated HSD17B2 mRNA. Because the high activity of HSD17B2 can decrease the active forms of androgens, this might imply a feed-back loop existing in the prostate for local control of androgen levels. We also found that DHT significantly up-regulated another androgen inactivating enzyme, HSD17B4 but significantly inhibited HSD17B5. In the prostate, the HSD17B5 enzyme seems to prefer DHT and androstenedione as substrates and favors the inactivation of androgen (Penning et al., 2001). In addition to the up-regulation of HSD17B2, calcitriol induced HSD17B4 and HSD17B5. In contrast to DHT, LXR agonist inhibited HSD17B2 but induced HSD17B5 expression. Moreover, it was able to completely block the effect of calcitriol on HSD17B2 induction. Therefore, it is possible that the effect of calcitriol on sex steroid hormone levels in prostate cancer can be modified by LXR. There are few studies concerning the relationship between LXR and sex steroid hormones. One study showed androgen concentrations were lower in LXR null mice (Frenoux et al., 2004) and another one (Robertson et al., 2005) reported intratesticular concentrations of testosterone, progesterone, and androstenedione in the LXR-/- testis were lower in mutant mice.

Taken together, the final output on androgen levels is thus a complex event, but the androgen effect on HSD17B2 might be the most significant. In comparison to androgen, mild contribution is from vitamin D, which in turn, is affected by LXR signaling.

Next we took a closer look at the cross-talk between VDR ligand and LXR agonist. Calcitriol not only decreased basal levels of ABCA1 expression, but also inhibited the LXR agonist, TO-901317, mediated induction of ABCA1. On the other hand, TO-901317 increased both basal and calcitriol induced CYP24 mRNA expression. The increase of CYP24 mRNA by TO-901317 was not due to an increase of VDR. Calcitriol had no effect on LXRα and LXRβ expression, suggesting that the inhibition of ABCA1 by calcitriol is not due to the down-regulation of these two transcription factors. Previously, Takahide M et al. (Jiang et al., 2006) showed that in H4IIE rat hepatoma cells $1\alpha,25(OH)_2D_3$ blunted the LXRα-mediated mRNA induction of cholesterol 7alpha-hydroxylase, a rate-limiting enzyme in the catabolism of cholesterol to bile acids (Hubacek and Bobkova, 2006; Shefer et al., 1970) which in turn is stimulated by oxysterol receptor, LXRα (Chiang, Kimmel, and Stroup, 2001; Peet et al., 1998). Thus, it appears that VDR negatively regulates LXR mediated induction of ABCA1 as well as cholesterol 7alpha-hydroxylase, which are all involved in cholesterol efflux and bile acid synthesis. Moreover, we found that TO-901317 slightly but significantly inhibited VDR expression whereas up-regulated CYP24, an enzyme responsible for inactivating of active vitamin D metabolites (Chen, Prahl, and DeLuca, 1993; Ohyama et al., 1993) and it is up-regulated by VDR (Chen and DeLuca, 1995). This suggests that LXR agonist might play a role in the negative regulation of the actions of VDR ligand. Interestingly, our *in vivo* study showed that in male the liver tissues of VDR-KO mice expressed significantly higher levels of LXRβ mRNA compared to that of WT mice. This implies that a functional VDR may repress LXRβ expression, which is in agreement with above hypothesis. Moreover, the LXRβ expression level difference was only observed in male mice, implying an involvement of AR and/or estrogen receptor.

Stimulated by a growing body of preclinical evidence that vitamin D inhibits the proliferation of prostate cancer, clinical trials with calcitriol in prostate cancer patients have been carried out since the 1990s (Beer, 2003). In the present study, we showed that TO-901317 inhibited LNCaP cell proliferation. Earlier Liao et al. (Fukuchi et al., 2004a) found that knockdown of ABCA1 expression by RNA interference in androgen-dependent cells increased their rate of proliferation and thus they proposed a potential of use of LXR signaling as a therapeutic target in prostate and other cancers (Chuu et al., 2007). Our present study suggests a mutual negative regulation of the actions of the ligands of VDR and LXR. Moreover, both calcitriol and TO-901317 regulate androgen activity regulatory enzymes, either with the same direction of DHT or the opposite direction. Given the important role of androgen in the development of prostate cancer, there can only be cautious suggestions for the use of activators of VDR or LXR signaling as a therapeutic option in prostate cancer

9.3 Studies with VDR deficient mice (IV)

Our previous study showed that vitamin D up-regulated cholesterol 25-hydroxylase (CH25H), an enzyme responsible for production of 25-hydroxycholesterol, which inhibits the synthesis of cholesterol (Adams et al., 2004). Therefore we were interested to study the effect of VDR on serum cholesterol level. VDR knockout mice were used as the model, in which serum cholesterol and HDL-C as well as cholesterol metabolism associated genes were examined in WT and KO mice. The total cholesterol was higher in mutant mice for both female and male although the QRT-PCR failed to detect lower CH25H mRNA in mutant liver due to its highly fluctuate expression pattern for individual mice. Future studies with increased sample number might be helpful. On the other hand, we observed the up-regulation of CH25H in human prostate stromal cells. When using human hepatocellular liver carcinoma cells, HepG2, we did not see effects of calcitriol on CH25H. This suggests that VDR mutation may have no effect on liver CH25H mRNA expression at all. This might explain why the present study did not detect any changes in ABCA1 expression, given the product of CH25H, 25-hydroxycholesterol, can act as a ligand of LXR and thus induce the expression of ABCA1. Interestingly, another gene, SREBP2, was found decreased in KO female mice but not in male mice. However, decrease of this protein is supposed to be in accordance with lowering of cholesterol, because SREBP2 is a transcription factor for activation of genes whose products play roles in the synthesis of cholesterol (Horton et al., 2003). A literature search revealed that an positive vitamin D response element has been reported in the promoter of murine Insig-2 (Lee et al., 2005), which is an endoplasmic reticulum retention protein that abrogate movement of the complexed SREBP to the Golgi apparatus for SREBP activation (Adams, Goldstein, and Brown, 2003; Brown et al., 2002; Feramisco et al., 2005; Radhakrishnan et al., 2004). This means that a decrease of Insig-2 favors an increase of SREBP2 activation of genes for cholesterol production. If this theory applies in real life, when the relative insig-2 decrease resulted in more free-SREBP2 with even less SREBP2 molecules, it is possible to increase the total cholesterol. Thus, it is of interest to test the expression of Insig-2 in future studies.

In male KO mice, however, SREBP2 was not significantly different from the WT. This suggests that gender plays a role in the control of cholesterol homeostasis, which is confirmed by the male specific changes of another two genes, LXRβ and ApoAI. In male mutant mice, liver LXRβ and ApoAI are higher than that of WT. LXRs function as cholesterol sensors as well as regulators of genes for cholesterol efflux and lipid transport to maintain cholesterol homeostasis (Naik et al., 2006; Repa and Mangelsdorf, 2000; Tangirala et al., 2002; Tontonoz and Mangelsdorf, 2003). Major cholesterol-related targets of LXRs include the ATP-binding cassette transporter family members such as ABCA1 (Costet et al., 2000; Repa et al., 2000), which mediates the efflux of excess cholesterol to acceptors such as HDL and ApoAI (Ohashi et al., 2005) for further processing and eventually of being eliminated. Thus the increase of liver LXRβ and ApoAI may lead to increase of HDL-C. Indeed, data from male mice showed significant increased HDL-C in the mutant group. It has been shown that over-expression of ABCA1 in the liver is associated with increased HDL-C level in transgenic mice and ABCA1 knockout leads to HDL-C deficiency (Singaraja et al., 2006). However, our data didn't show any significant change of liver ABCA1 mRNA expression levels in mutant mice,

especially in males in which mutant mice had higher HDL-C levels. This indicates ABCA1 is not involved in the changed lipid profile in mutant mice. Interestingly, male mutant mice have higher level of LXRβ mRNA expression. Given the role of LXRβ as cholesterol sensors and transcription factors for activating not only ABCA1, but also ABCG1 (Kennedy et al., 2001), ABCG5 (Berge et al., 2000) and ABCG8 (Repa et al., 2002), it is possible that the LXRβ exerts its effects on HDL-C levels by activating other cholesterol exporters. It is worth testing the expression of these transporters in future studies.

The statements above are based on the assumption that knockout of the VDR affected gene expression pattern, which in turn, led to an altered lipid profile. One might argue that it might happen the other way around, which is altered lipid profile resulted from VDR knockout causes the changes of the gene expression pattern. It is possible. However, it has been reported that calcitriol down-regulated ApoAI mRNA and protein in the human hepatoma cell line HepG2, and both VDR and VDRE on the promoter of ApoAI are required (Wehmeier et al., 2005). This provides a support for the former assumption.

It is worthy to note that the results discussed above are from strain 129S1, in which mutant mice were fed a special diet containing 2% Ca, 1.25% P and 20% lactose to normalize their mineral metabolism. In our experiments using NMRI mice, both WT and KO mice were fed with special food three weeks before sacrifice. We failed to observe a significant difference in cholesterol and/or HDL-C level for WT and mutant mice. It remains unclear whether this is because of the effects of the food or the strain of the mice. However, the gender difference within both wild-type and mutant mice were again observed. Future studies by using the same strain of mice fed with the same food before sample collection will be helpful.

In conclusion, our study suggests that the lack of functional VDR may lead to increased cholesterol in both male and female mice, and HDL-C in male mice only. This is possible via altered cholesterol metabolism related gene expression such as ApoAI, LXRβ and cholesterol production related gene like SREBP2. There is evidence suggesting that vitamin D deficiency contributes to cardiovascular diseases such as atherosclerosis (Zittermann, 2003). Apparently, it is worth doing more comprehensive studies on the lipid metabolism employing the VDR-KO model.

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12. ORIGINAL COMMUNICATIONS

CORRIGENDA

There are three errors in the original communication:

Article I

1. P. 721: Methods / *Cell growth assay*, line 4, “fixed with 11% gultaraldehyde” should read “fixed with 1% glutaraldehyde”
2. P. 723: Results / *Effects of CH25H inhibitor and 25-hydroxycholesterol on P29SN cell proliferation* / 2nd paragraph / last sentence, “(P < 0.05)” should read “(P > 0.05)”

Article III

1. P. 103: Results/3.7. Effect of calcitriol on HSD17B2, HSD17B4 and HSD17B5 mRNA expression/last sentence, “HSD17B5” should read “HSD17B4”.

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Regulation of cholesterol 25-hydroxylase expression by vitamin D_3 metabolites in human prostate stromal cells ☆

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Abstract

Vitamin D_3 plays an important role in the control of cell proliferation and differentiation. Cholesterol 25-hydroxylase (CH25H) is an enzyme converting cholesterol into 25-hydroxycholesterol. Vitamin D_3 as well as 25-hydroxycholesterol has been shown to inhibit cell growth and induce cell apoptosis. Here we show that 10 nM $1\alpha,25(OH)_2D_3$ and 500 nM $25OHD_3$ upregulate CH25H mRNA expression in human primary prostate stromal cells (P29SN). Protein synthesis inhibitor cycloheximide does not block $1\alpha,25(OH)_2D_3$ mediated upregulation of CH25H mRNA. Transcription inhibitor actinomycin D blocks basal level as well as $1\alpha,25(OH)_2D_3$ induced CH25H mRNA expression. $1\alpha,25(OH)_2D_3$ has no effect on CH25H mRNA stability. 25-Hydroxycholesterol significantly decreased the P29SN cell number. A CH25H enzyme inhibitor, desmosterol, increases basal cell number but has no significant effect on vitamin D_3 treated cells. Our data suggest that *ch25h* could be a vitamin D_3 target gene and may partly mediate anti-proliferative action of vitamin D_3 in human primary prostate stromal cells.

Keywords: Calcitriol; Calcidiol; Cholesterol 25-hydroxylase; Gene regulation; Cell proliferation

The vitamin D_3 receptor is a nuclear receptor and that binds with high affinity to calcitriol, $1\alpha,25(OH)_2D_3$ [1] and with a significantly lower affinity to calcidiol, $25OHD_3$ [2]. Both metabolites appear to regulate gene expression [3]. Vitamin D_3 metabolites appear to control cell growth via regulation of cell cycle, cell differentiation, and cell apoptosis [4,5]. It is possible that fatty acid and lipid metabolism are involved in vitamin D_3 action. Moreno reported that regulation of prostaglandin metabolism and biological actions constitutes a novel pathway of calcitriol action that may contribute to its anti-proliferative effects in prostate cells [6]. Qiao et al. [7] found that $1\alpha,25(OH)_2D_3$ inhibited fatty acid synthase expression in prostate cancer LNCaP cells while inhibition of the LNCaP cell growth also occurred. Vitamin D_3 may affect cholesterol metabolism. Vitamin D_3 deficiency has been reported to be associated with increased blood cholesterol concentrations [8]. A negative correlation of $25OHD_3$ concentration with total and LDL cholesterol has also been observed [9]. The mechanism of the relationship between vitamin D_3 and cholesterol metabolism is not known.

CH25H is an important enzyme of cholesterol metabolism. It belongs to a family of enzymes that utilize diiron cofactors to catalyse the hydroxylation of hydrophobic substrates such as cholesterol to 25-hydroxycholesterol [10]. 25-Hydroxycholesterol is one of the natural oxysterols. 25-Hydroxycholesterol inhibits the activation of SREBPs [11], which are transcription factors that activate genes involved in the synthesis of cholesterol and other lipids in animal cells [12]. Thus, 25-hydroxycholesterol causes

☆ *Abbreviations:* CH25H, cholesterol 25-hydroxylase; SREBP, sterol-regulatory element binding protein; $1\alpha,25(OH)_2D_3$,1α,25-dihydroxyvitamin D_3; $25OHD_3$,25-hydroxyvitamin D_3.

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a decreased cholesterol synthesis. Furthermore, 25-hydroxycholesterol seems to inhibit the growth of tumour as well as normal cells [13,14]. In addition, 25-hydroxycholesterol induces apoptosis through an inhibition of c-myc [15].

Our microarray data suggested that both $1\alpha,25(OH)_2D_3$ and $25OHD_3$ may up-regulate CH25H, therefore we were interested to study the relationship of CH25H and vitamin D_3 in more detail. We studied whether CH25H participates in the anti-proliferative action of vitamin D_3 in human primary prostate stromal cells.

Methods

Reagents. $1\alpha,25(OH)_2D_3$, $24R,25(OH)_2D_3$, and $25OHD_3$ were obtained from Leo Pharmaceuticals (Ballerup, Denmark) and DHT was from Merck (Darmstadt, Germany). Cycloheximide, actinomycin D, desmesterol (5, 24-cholestadien-3β-ol), 25-hydroxycholesterol (cholest-5-ene-3β, 25-diol), and RPMI-1640 medium were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). FBS was purchased from Gibco-BRL (Life Technology, Paisley, Scotland). TRIzoL reagent was purchased from Invitrogen (Carlsbad, USA). High Capacity DNA Archive Kit and SYBR Green PCR Master Mix Kit were purchased from Applied Biosystems (Foster City, USA). One-Cycle Target Labelling Assay Kit and Human Genome U133 Plus 2.0 GeneChip were purchased from Affymetrix (Affymetrix, Inc. Santa Clara, CA).

Cell treatment and RNA isolation. Human primary prostate stromal cells termed P29SN [3] were maintained in phenol red-free DMEM/F12 medium, supplemented with 10% FBS, 3 mM L-glutamine, 5 μg/ml insulin, and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) at 37 °C in a humid atmosphere with 5% CO_2. Twenty four hours before treatment the medium was changed to 10% FBS-DCC (10% FBS treated with dextran-coated charcoal) supplemented DMEM/F12 medium. Cells were treated with vitamin D_3 metabolites or/and other reagents, which were diluted in 100% ethanol. Total cellular RNA was isolated with TRIzoL reagent following the instructions from the manufacturer. The RNA concentration was calculated from absorbance at 260 nm in a GeneQuant II (Pharmacia Biotech, USA) and A280/A260 was measured to verify the purity of the RNA. The ratio of all the RNA samples fell in 1.9–2.1. Randomly selected RNA samples were subjected to denaturing-gel electrophoresis. The ratio of the intensity of the 28S band and that of the18S band was 1.5–2.0.

Affymetrix microarray analysis. cRNA preparation was performed following the instructions of the manufacturer. Labelled cRNA was hybridized on GeneChip Human Genome U133 Plus 2.0 Array representing over 47,000 transcripts. Scanned images were processed using Affymetrix GeneChip Operating Software Server 1.0 (GCOS Server) (Affymetrix, Santa Clara, CA) and raw data were normalized and analysed using GENESPRING software (Version 7.2, Silicon Genetics, Redwood City, CA).

Real-time PCR analysis and primer design. Real-time PCR was performed in ABI PRISM 7000 Detection System (Applied Biosystems, USA). Total cellular RNA (3–5 μg) was used to synthesize cDNA by using High Capacity Archive Kit (Applied Biosystems, USA) in a final volume of 100 μl. Undiluted cDNA (1–2 μl) reactions or 4–8 μl of 1:10 diluted cDNA reactions was used as input for each of the real-time quantitative PCR reactions by using SYBR Green PCR Master Mix kit (Applied Biosystems, USA). Primers were designed by using Primer Express v2.0 software (Perkin-Elmer Applied Biosystems, Foster City, CA). BLASTn searches were performed to ensure that the primers were gene specific. Since human CH25H gene spans one exon, real-time PCR using unreverse-transcribed RNA as template was performed to confirm that the experimental results were not compromised through genomic DNA contamination. CH25H (NM_003956) forward primer was 5′-TCCTGTTC TGCCTGCTACTCTTC-3′ and its reverse primer was 5′-GGTACAGC CAGGGCACCTT-3′. CYP24 (NM_000782) forward primer was 5′-GCC CAGCCGGGAACTC-3′ and its reverse primer was 5′-AAATACCA CCATCTGAGGCGTATT-3′. CYP27A1(M62401) forward primer was 5′-GAGTGGACACGACATCCAACAC-3′ and its reverse primer was 5′-CTCCTGGATCTCAGGGTCCTT-3′. Human housekeeping gene RPLP0 (NM_001002) was used as endogenous control. Primers for RPLP0 were as follows, forward: 5′-AATCTCCAGGGGCACCATT-3, reverse: 5′-CGCTGGCTCCCACTTTGT-3′.

Cell growth assay. P29SN cells were treated with hormones and other reagents for 0, 3, 6, and 9 days. Eight experiments for each treatment were repeated three times. Cell growth was analysed with crystal violet staining as described earlier [16]. Briefly, cells were fixed with 11% gultaraldehyde for 15 min by shaking at 500 rpm, washed, and air-dried completely. Crystal violet solution (0.1%) was added to stain the fixed cells for 20 min with shaking at 500 rpm. Excess dye was removed by extensive washing with tap water. The plates were air-dried. 10% acetic acid was used to withdraw cell-bound dye. The optical density of extracted dye was measured in plates at 590 nm by Microplate Reader (Wallac, victor 1420 multilabel counter, Turku, Finland).

Statistical analysis. Data of real-time PCR are expressed as mean values ± SD. Significance was assessed by using Student's paired t test otherwise indicated. $^{*}P < 0.05$ was considered as significant, $^{**}P < 0.001$ as highly significant and $P > 0.05$ as not significant (NS).

Results

Vitamin D_3 increases CH25H mRNA expression

To investigate vitamin D_3 regulated genes in human primary prostate stromal cells, Affymetrix microarray analysis was performed using Human Genome U133 Plus 2.0 Array chip representing over 47,000 transcripts, which include 38,500 well-characterized human genes. Cholesterol 25-hydroxylase was shown to be upregulated by 10 nM $1\alpha,25(OH)_2D_3$ and 500 nM $25OHD_3$ in human primary prostate stromal cells. Quantitative real-time PCR results showed that 10 nM $1\alpha,25(OH)_2D_3$ and 500 nM $25OHD_3$ treatment for 24 h increased CH25H mRNA expression

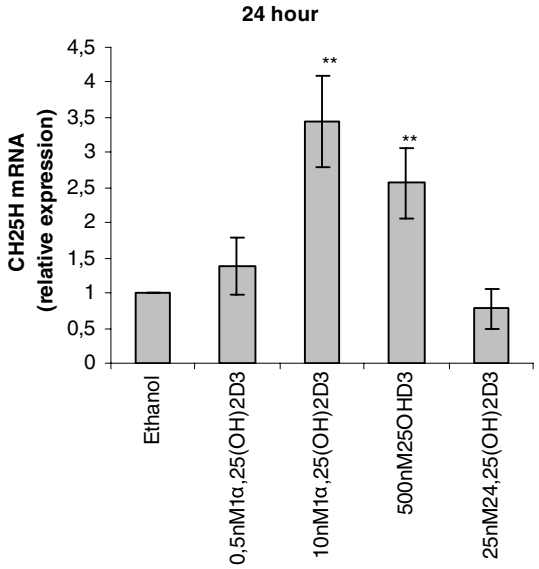

Fig. 1. Up-regulation of CH25H mRNA expression by vitamin D_3 metabolites in human primary prostate stromal cells. Cells were treated with 0.1% ethanol and different Vitamin D_3 metabolites at various concentrations as indicated for 24 h. Relative CH25H mRNA expression was analysed by quantitative real-time PCR. ($n = 4$, $^{**}P < 0.001$).

level 3.43 ± 0.65 ($P < 0.001$) and 2.57 ± 0.51 ($P < 0.001$) fold, respectively, 0.5 nM $1\alpha,25(OH)_2D_3$ and 25 nM $24,25(OH)_2D_3$ had no significant effects on CH25H expression (Fig. 1).

The time course studies showed that inductions of CH25H mRNA by 10 nM $1\alpha,25(OH)_2D_3$ and 500 nM $25OHD_3$ reached maximum at 12 h and remained high until 48 h (Fig. 2). Dose dependency studies demonstrated that CH25H expression was 2.57 ± 0.51 ($P < 0.001$) fold enhanced by 500 nM $25OHD_3$, but not changed at 50, 100, and 250 nM.

Studies with cycloheximide and actinomycin D

To study whether the up-regulation of CH25H mRNA required protein synthesis, we pre-treated P29SN cells with protein synthesis inhibitor cycloheximide (10 μg/ml) for 30 min followed by ethanol (0.2%) or $1\alpha,25(OH)_2D_3$ (10 nM) treatment for 21 h. It seems that cycloheximide had no significant effect on calcitriol mediated CH25H mRNA induction (Fig. 3.)

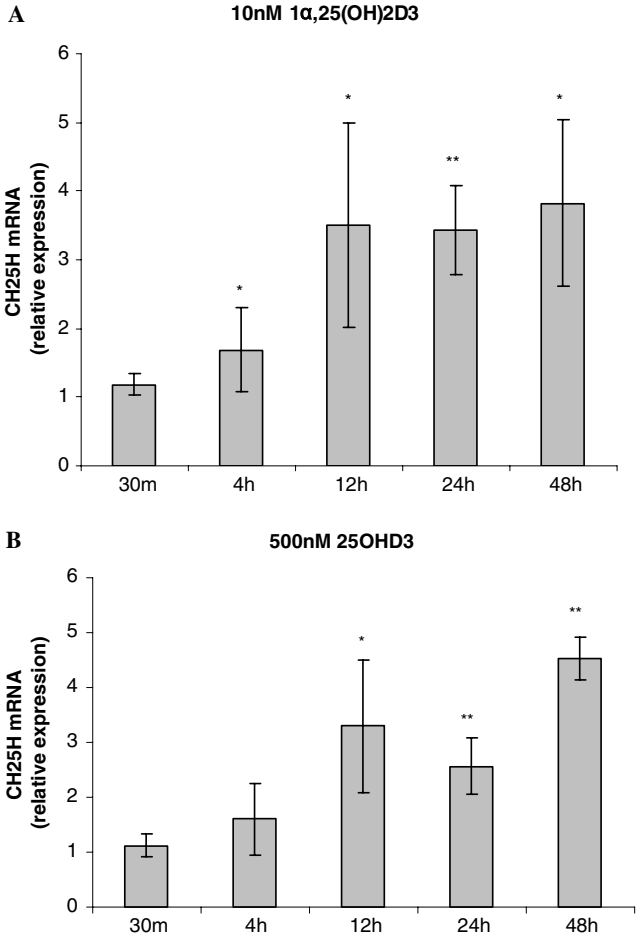

Fig. 2. Time course of CH25H mRNA induction after vitamin D_3 metabolites. (A) Cells were treated with 10 nM $1\alpha,25(OH)_2D_3$ (A) or 500 nM 25OHD3 (B) for 30 min, 4, 12, 24, and 48 h. ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.001$).

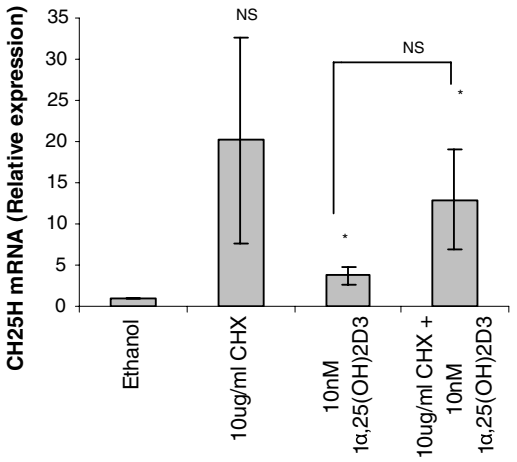

Fig. 3. Effect of cycloheximide (CHX) on CH25H mRNA expression. CHX has no significant effect on vitamin D_3 mediated CH25H induction. P29SN cells were treated with 10 μg/ml CHX and10 nM $1\alpha,25(OH)_2D_3$ alone or in combination for 24 h. ($n = 3$, $^{*}P < 0.05$).

We next determined whether the increase in CH25H mRNA was due to increased stability of the CH25H mRNA in the presence of calcitriol. First, cells were treated with 0.2% ethanol, 10 nM $1\alpha,25(OH)_2D_3$, 5 μg/ml transcription inhibitor actinomycin D alone or in combination with 10 nM $1\alpha,25(OH)_2D_3$ for 24 h. Both basal and calcitriol mediated upregulation of CH25H mRNA were blocked by act D treatment (Fig. 4A.), indicating that calcitriol mediated upregulation of CH25H is transcription dependent. Second, we pre-treated cells with 0.1% ethanol or 10 nM $1\alpha,25(OH)_2D_3$ for 21 h followed by incubation in the presence of act D for 0, 2, 4, and 8 h (Fig. 4B). Fig. 4C presents the data transformed from Fig. 4B and analysed by GraphPad Prism software (GraphPad Prism version 4.03 for Windows, www.graphpad.com), in both calcitriol-treated and untreated cases, CH25H mRNA degraded quickly after act D treatment. The mean half-life of CH25H mRNA in ethanol and hormone treated P29SN cells was 2.9 and 2.5 h, respectively, which were retrieved from corresponding best-fit-in curves with slopes -10.20 ± 3.998 ($r^2 = 0.7650$) and -10.36 ± 4.692 ($r^2 = 0.7092$), respectively. The difference between the two slopes and hence the half-lives was not significant with $P = 0.9806$ (Fig. 4C).

Effect of CH25H inhibitor and 25-hydroxycholesterol on P29SN cell proliferation

To determine whether P29SN cell proliferation was inhibited by 25-hydroxychloesterol, we treated P29SN cells with 25-hydroxycholesterol at 0.01, 0.1, 1.0, and 10 μg/ml for 0, 3, 6, and 9 days. At concentrations of 0.01 and 0.1 μg/ml, 25-hydroxycholesterol had no effect on P29SN cell proliferation. However, at concentrations of 1.0 and 10 μg/ml it significantly inhibited cell proliferation at day 3, 6, and 9 ($^{*}P < 0.05$, $^{**}P < 0.001$) (Fig. 5A).

We wanted to determine whether the upregulation of CH25H by calcitriol contributes to its anti-proliferation

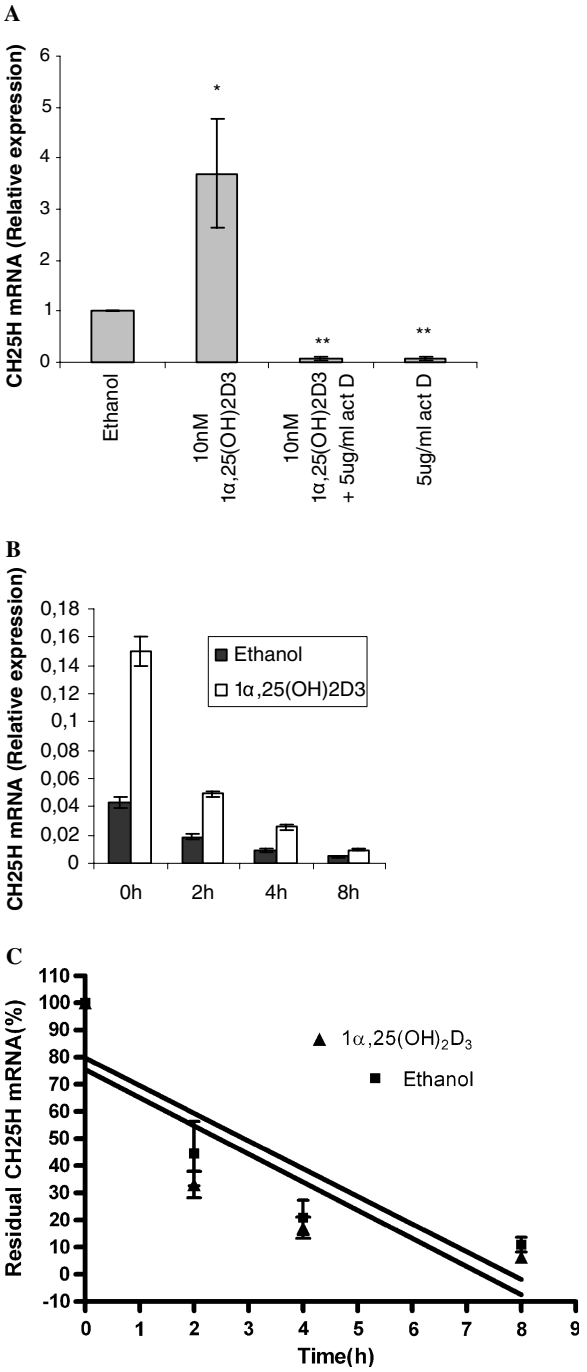

Fig. 4. Effect of actinomycin D (act D) on CH25H mRNA expression (A). Act D inhibits calcitriol induced CH25H mRNA expression. P29SN cells were treated with 5 μg/ml act D and 10 nM $1\alpha,25(OH)_2D_3$ alone or in combination for 24 h. Relative CH25H mRNA level was determined by RT-PCR. ($n = 3$, $^{*}P < 0.05$ and $^{**}P < 0.001$). (B,C) CH25H mRNA stability. P29SN cells were pre-stimulated with 0.1% ethanol or 10 nM $1\alpha,25(OH)_2D_3$ for 21 h followed by act D treatment for 0, 2, 4, and 8 h. Relative CH25H mRNA level was determined by RT-PCR. Data shown represents the average of three independent experiments (B) and display the linear regression of CH25H mRNA degradation (C).

in P29SN cells. P29SN cells were treated with 10 nM $1\alpha,25(OH)_2D_3$ and 30μM desmosterol [10], alone or in combination for 0, 3, 6, and 9 days. At day 3, 6, and 9 10 nM $1\alpha,25(OH)_2D_3$ significantly inhibited cell proliferation ($P < 0.05$) (Fig. 5B). Desmosterol significantly increased cell proliferation at day 9 ($P < 0.05$) (Fig. 5B). The effect of desmosterol on calcitriol mediated anti-proliferation was under the limit of detection ($P < 0.05$) (Fig. 5B).

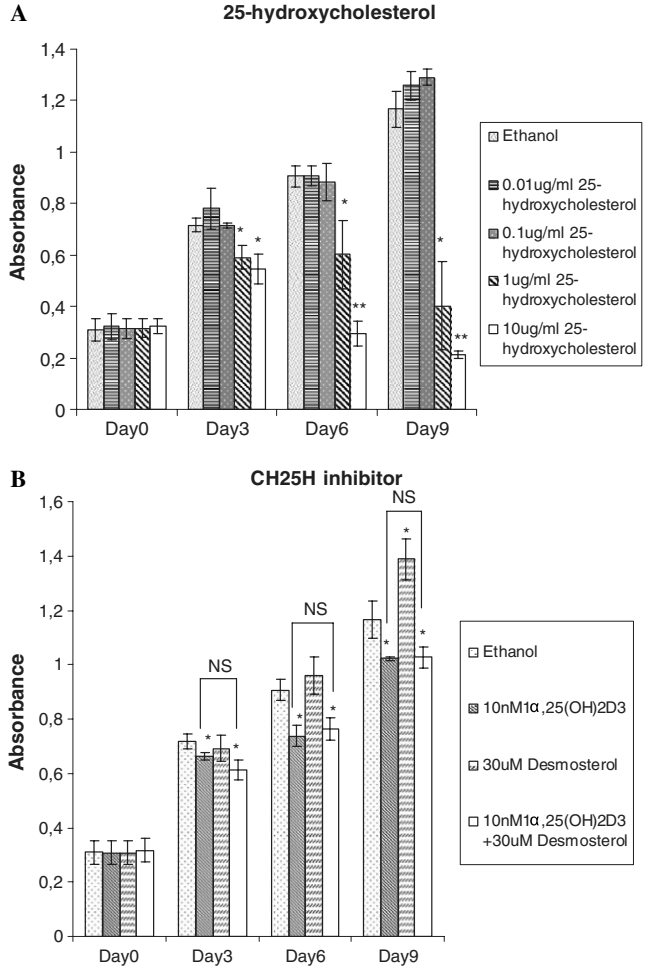

Fig. 5. Effect of desmosterol and 25-hydroxycholesterol on P29SN cell proliferation. P29SN cells treated with 25-hydroxycholesterol, $1\alpha,25(OH)_2D_3$, and/or desmosterol for 0, 3, 6, and 9 days. Relative cell number was analysed with crystal violet assay ($n = 24$, $^{*}P < 0.05$ and $^{**}P < 0.001$). (A) 25-Hydroxycholesterol at 1 and 10 μg/ml significantly decreased cell proliferation at days 3, 6, and 9. (B) At day 9, desmosterol treated wells show significantly higher cell number compared with ethanol treated wells. There was no significant difference between hormone treated and hormone plus desmosterol treated wells.

Cholesterol 27-hydroxylase (CYP27A1) expression

Because 25-hydroxycholesterol can be produced by CYP27A1 [26], we studied its possible regulation by calcitriol. 10 nM $1\alpha,25(OH)_2D_3$ has no significant effect on CYP27A1 mRNA expression (Fig. 6).

25-Hydroxyvitamin D_3-24-hydroxylase (CYP24) expression

To confirm that P29SN cells were stimulated successfully by vitamin D_3 metabolites, CYP24 mRNA expression

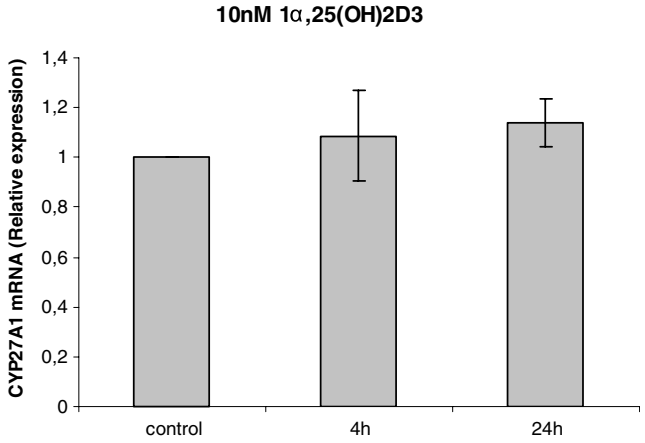

Fig. 6. Effect of calcitriol on CYP27A1 mRNA expression. Calcitriol treatment has no significant effect on CYP27A1 expression. P29SN cells were treated with 10 nM $1\alpha,25(OH)_2D_3$ for 4 and 24 h. ($n = 3$).

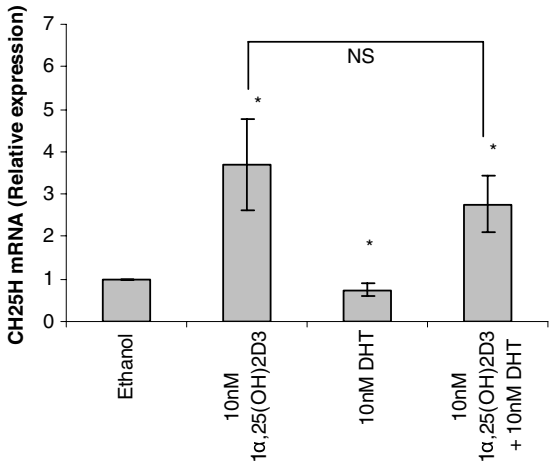

Fig. 7. Effect of 5α-dihydrotestosterone (DHT) on CH25H mRNA expression. DHT treatment has no effect on calcitriol induced CH25H expression. P29SN cells were treated with 10 nM/ml DHT and 10 nM $1\alpha,25(OH)_2D_3$ alone or in combination for 24 h. Relative CH25H mRNA level was determined by RT-PCR. ($n = 3$, $^*P < 0.05$).

was examined. At 24 h treatment, 10 nM calcitriol increased CYP24 mRNA to 3100 ± 290-fold ($n = 3$, $P < 0.001$), suggesting the P29SN cells were responsive to vitamin D_3 treatment.

Effect of DHT

DHT had no effect on calcitriol induced CH25H mRNA expression (Fig. 7).

Discussion

$25OHD_3$, $1\alpha,25(OH)_2D_3$, and $24,25(OH)_2D_3$ are the principal circulating metabolites of vitamin D_3 in the plasma. The present study showed for the first time that, $1\alpha,25(OH)_2D_3$ and $25OHD_3$, can upregulate cholesterol 25-hydroxylase in human primary prostate stromal cells. At the concentration of 250 nM, $25OHD_3$ has been shown to induce 25-hydroxyvitamin D_3 24-hydroxylase (CYP24) [3], a gene which is highly sensitive to vitamin D_3 [17,18]. In the present study, 500 nM $25OHD_3$, was needed for the increased CH25H mRNA expression. This concentration is only slightly (2- to 5-fold) above the physiological one. In contrast, 100-fold concentration above the physiological one, 10 nM $1\alpha,25(OH)_2D_3$ was needed for corresponding induction of CH25H. In prostate stromal cells, the expression level of 25-hydroxyvitamin D_3-1α-hydroxylase, the enzyme for converting $25OHD_3$ into $1\alpha,25(OH)_2D_3$, is extremely low. Therefore, $25OHD_3$ may directly regulate CH25H mRNA expression. This is in agreement with our earlier report [3].

To study whether protein synthesis is needed for the regulation of CH25H by vitamin D_3, a protein synthesis inhibitor, cycloheximide, was used. CHX did not affect significantly CH25H mRNA induced by calcitriol and CH25H mRNA stability was not affected by calcitriol, suggesting that *ch25h* might be regulated directly by vitamin D_3.

25-Hydroxycholesterol inhibits the synthesis of cholesterol [11,19,20]. Cholesterol synthesis is the prerequisite for cell proliferation [21]. Several studies clearly demonstrate that calcitriol is involved in the control of cell proliferation [4,5]. Therefore, we were interested to study whether CH25H induced by calcitriol contributes to the anti-proliferative action. First, we tested whether 25-hydroxycholesterol can inhibit P29SN cell growth. Indeed, 25-hydroxycholesterol seemed significantly to decrease prostate cell number. The decreased cell number by 25-hydroxycholesterol during incubation over 6 and 9 days may be due to increased apoptosis [22]. Next, we used a cholesterol 25-hydroxylase inhibitor, desmosterol, to treat P29SN cells, alone or in combination with calcitriol. The results showed that desmosterol promoted basal cell proliferation significantly at day 9, implying that the inhibition of basal level of cholesterol 25-hydroxylase promoted the growth of the cells. We did not observe an effect of desmosterol on calcitriol exerted growth inhibition. It was possible that the dosage of desmosterol used in this study could inhibit the basal level of the cholesterol 25-hydroxylase activity but was not high enough to inhibit calcitriol up-regulated enzyme activity.

Vitamin D_3 level seems to be inversely related to serum cholesterol levels [8,9]. However, the mechanism behind this phenomenon is not clear. 25-Hydroxycholesterol is a potent inhibitor of cholesterol synthesis [11,20], and therefore, our finding of upregulation of CH25H by vitamin D_3 could at least partially explain how vitamin D_3 can control serum cholesterol level. We have preliminary data suggesting that VDR knock-out mice have an increased total serum cholesterol concentration (Wang et al., unpublished).

In prostate, vitamin D_3 inhibits cell growth by androgen dependent and independent mechanisms [23–25]. Because we used DCC-FBS (androgen depleted) medium during

the incubations, the up-regulation of CH25H by calcitriol was apparently androgen-independent. This was confirmed by the fact that 5α-dihydrotestosterone (DHT) addition had no significant effect on vitamin D regulation of CH25H mRNA.

In conclusion, this study provides the first evidence that vitamin D_3 may directly upregulate cholesterol 25-hydroxylase. Through 25-hydroxycholesterol, vitamin D_3 might regulate serum cholesterol concentration. Since 25-hydroxycholesterol inhibited prostate cell growth and cholesterol 25-hydroxylase inhibitor, desmosterol, enhanced basal cell proliferation, we propose that vitamin D_3 mediated anti-proliferation may be partially due to the upregulation of cholesterol 25-hydroxylase.

Acknowledgments

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Regulation of 17β-hydroxysteroid dehydrogenase type 2, type 4 and type 5 by calcitriol, LXR agonist and 5α-dihydrotestosterone in human prostate cancer cells

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Abstract

Vitamin D seems to be involved in the control of prostate cancer cell growth. 17β-Hydroxysteroid dehydrogenases type 2, type 4 and type 5 are enzymes which regulate intracellular concentration of active sex steroid hormones, which in turn, regulate the development, growth, and function of the prostate and play a role in the development and progression of prostate cancer. Using quantitative real-time PCR we find that calcitriol up-regulates HSD17B type 2, type 4 and type 5 in human prostate cancer LNCaP and PC3 cells but not in stromal cells. LXR agonist, TO-901317, suppresses the expression of HSD17B2 mRNA and inhibits calcitriol induced HSD17B2 expression. TO-901317 up-regulates the expression of HSD17B5 but not that of HSD17B4. 5α-Dihydrotestosterone up-regulates the expression of HSD17B2 and HSD17B4 but it significantly inhibits HSD17B5 expression by 70%. Calcitriol has no effect on DHT mediated expression of the three genes. The regulation of HSD17B2, HSD17B4 and HSD17B5 by ligands of LXR and VDR as well as AR in prostate cancer cells suggests a complex interaction of these signaling systems in the prostate.

Keywords: HSD17B2; HSD17B4; HSD17B5; Calcitriol; VDR; LXR; AR; Gene regulation and prostate cancer

1. Introduction

The human *HSD17B* gene family encodes for the 17β-hydroxysteroid dehydrogenases that play a pivotal role in the production of steroid hormones such as androgen, among which HSD17B2 was identified as a membrane-bound enzyme which preferentially catalyzes the oxidation of the 17β-hydrxyl group of estradiol-17β and testosterone as well as the 20α-hydroxyl group of 20α-dihydroprogesterone [1]. It is expressed in different tissues such as breast, uterus, testis, liver, prostate and especially highly expressed in placenta [2–5]. Estradiol, progesterone and activin regulation of 17β-hydroxysteroid dehydrogenases has been reported [6–8]. Hughes et al. [9] found that calcitriol up-regulated HSD17B2 at both mRNA and enzyme activity levels in cultured keratinocytes. Their work suggested that HSD17B2 might be involved in skin cancer development. Interestingly, an association of the loss of heterozygosity at HSD17B2 gene containing chromosomal region, 16q24.1–16q24.2, and a risk for clinically aggressive prostate cancer has been reported [2,10,11]. The expression level of HSD17B2 is significantly

Abbreviations: 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; HSD17B2, 17β-hydroxysteroid dehydrogenase-2; HSD17B4, 17β-hydroxysteroid dehydrogenase-4; HSD17B5, 17β-hydroxysteroid dehydrogenase-5; VDR, vitamin D$_3$ receptor; LXR, liver X receptor; AR, androgen receptor; DHT, 5α-dihydrotestosterone

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higher in benign prostatic hyperplasia compared with the carcinoma specimens [12]. Vitamin D deficiency is a risk factor for prostate cancer mortality [13–15]. Therefore, we were interested to study the effect of calcitriol on the expression of HSD17B2 in human prostate cancer cells and stromal cells.

In addition to HSD17B2, there are other enzymes from the same family which regulate cellular androgen level, which, in turn, plays critical roles in prostate cancer development. HSD17B4 is ubiquitously expressed in a wide variety of tissues and catalyses oxidative reactions such as conversion of testosterone into androstenedione [16]. HSD17B5 (AKR1C3) is expressed in the liver, endometrium, ovary, prostate and mammary gland [17,18] and it acts as a 17-ketosteroid reductase and converts androstenedione into testosterone. However, in the prostate the enzyme seems to prefer DHT and androstenedione as substrates and thus favors the inactivation of highly active DHT [17]. It was reported that HSD17B4 expression was abolished after vitamin D-propagated differentiation in human THP1 myeloid leukemia cells [19], but in prostate cells it seems there are no report concerning the effect of calcitriol on HSD17B4 or HSD17B5. Therefore, we have examined the effects of calcitriol on the expression of the two genes.

Androgens are involved in prostate cancer development [20,21]. There is no study concerning the effect of androgen on the expression of HSD17B2 although there are studies indicating that changes of HSD17B2 activity result in changes of androgen levels [22–24], thus we studied the effect of active androgen on the expression of HSD17B2 as well as HSD17B4 and HSD17B5. There seems to be interaction between LXR and androgen levels [25], therefore we aimed to analyse the interactions between ligands of VDR, AR and LXR.

2. Materials and methods

2.1. Reagents

1α,25(OH)$_2$D$_3$ was obtained from Leo Pharmaceuticals (Ballerup, Denmark). DHT was purchased from Merck (Ballerup, Denmark). TO-901317 and RPMI-1640 medium were purchased from Sigma–Aldrich (Saint Louis, MO, USA). FBS was purchased from Gibco-BRL (Life Technology, Paisley, Scotland). TRIzoL reagent was purchased from Invitrogen (Carlshad, USA). High Capacity DNA Archive Kit and SYBR Green PCR Master Mix Kit were purchased from Applied Biosystems (Forster City, USA).

2.2. Cell treatment and RNA isolation

Human prostate cancer cell lines LNCaP clone FGC, DU145 and PC3 (American Type Culture Collection) were cultured in phenol red-plus RPMI-1640 medium, supplemented with 10% FBS or 10% FBS–DCC, 5 μg/ml insulin and antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml) at 37 °C in a humid atmosphere with 5% CO_2. Human primary prostate stromal cells termed P29SN [26] were cultured in phenol red-free DMEM/F12 medium, supplemented with 10% FBS, 3 mM L-glutamine, 5 μg/ml insulin and antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml) at 37 °C in a humid atmosphere with 5% CO_2. For P29SN cells and LNCaP cells that were subjected to DHT treatment, the medium was changed to 10% FBS–DCC supplemented corresponding medium 24 h before the treatments. Cells were treated with calcitriol or/and other reagents, which were diluted in 100% ethanol. Cells were grown to 50% confluence for treatment. Total cellular RNA was isolated with TRIzoL reagent following the instructions from the manufacturer.

2.3. Quantitative real-time PCR

Quantitative real-time PCR were performed in ABI PRISM 7000 Detection System (Applied Biosystems, USA). cDNA was synthesised using High Capacity Archive Kit (Applied Biosystems, USA) and was used as template for real-time quantitative PCR using SYBR Green PCR Master Mix kit (Applied Biosystems, USA). Data was normalized by dividing the quantity of target gene by the quantity of a housekeeping gene, GAPDH. Another housekeeping gene, PBGD, was used to confirm the data. Data shown were normalized with GAPDH. Primers were designed using Primer Express v2.0 software (Perkin-Elmer Applied Biosystems, Foster City, CA). BLASTn searches were performed to ensure that—the primers were gene specific. Primers for each gene were as followings: GAPDH (NM_002046): forward, 5′-CCACATCGCTCAGACACCAT-3′; reverse, 5′-ACCAGGCGCCCAATACG-3′. HSD17B2 (BC059170): forward, 5′-GGCCATGCTTTGTGCAAGT-3′; reverse, 5′-TCATTCAAAACTCCGGCAAAT-3′. HSD17B4 (NM_000414): forward, 5′-TTGGGCCGAGCCTATGC-3′; reverse, 5′-CCCCTCCCAAATCATTCACA-3′. HSD17B5 (NM_003739): forward, 5′-GGGATCTCAACGAGACAAACG-3′; reverse, 5′-AAAGGACTGGGTCCTCCAAGA-3′. PBGD (X04808): forward 5′-CACACACAGCCTACTTTCCAA-3′; reverse, 5′-TTTCTTCCGCCGTTGCA-3′. RPLP0 (NM_001002): forward, 5′-AATCTCCAGGGGCACCATT-3; reverse, 5′-CGCTGGCTCCCACTTTGT-3′. Housekeeping gene, RPLP0, was control gene used to normalize data from P29SN cells.

2.4. Statistical analysis

Data of real-time PCR are expressed as the mean values ± S.D. Significance was assessed using the two-tail Student's paired *t*-test. $^{*}P < 0.05$ was considered as statistically significant, $^{**}P < 0.001$ as highly significant and $P > 0.05$ as not significant (NS).

3. Results

3.1. Basal expression of HSD17B2, HSD17B4 and HSD17B5

To study the basal expression level of 17β-hydroxysteroid dehydrogenase type 2, type 4 and type 5 mRNA in human prostate cancer cells, LNCaP, DU145 and PC3, quantitative real-time PCR was performed. The results demonstrate that in LNCaP cells, the mRNA expression level of the three types of enzyme differ clearly with high expression of type 4. In DU145 cells, type 4 and type 5 mRNA levels are similar but type 2 is very low. In PC3 cells, all the three enzymes have similar mRNA expression level. The relative expression of HSD17B2, HSD17B4 and HSD17B5 is normalized to housekeeping gene (GAPDH) and in LNCaP cells is 1-, 1907- and 35-fold, in DU145 cells is 0.6-, 750- and 1026-fold, in PC3 cells is 880-, 808- and 853-fold, respectively.

3.2. Effect of calcitriol on HSD17B2 expression

In the medium containing normal serum, calcitriol significantly up-regulates HSD17B2 mRNA expression in a dose (Fig. 1A) and time-dependent (Fig. 1B) manner in LNCaP and PC3 cells. At 100 nM, calcitriol up-regulates HSD17B2 mRNA 5.0- and 3.7-fold in LNCaP and PC3 cells, respectively (Fig. 1A). A maximal induction of HSD17B2 mRNA by 10 nM calcitriol occurs at 48 h in both LNCaP and PC3 cells (Fig. 1B). In the medium containing DCC-treated serum, calcitriol has no effect on HSD17B2 expression at 24 h treatment (data not shown).

3.3. Effect of LXR agonist (TO-901317) on HSD17B2 mRNA expression

At 24 h, 10 μM TO-901317 treatment of LNCaP and PC3 cells significantly decreases HSD17B2 mRNA expression by 91% and 30%, respectively (Fig. 2A and B). Furthermore, TO-901317 inhibits calcitriol induced expression of HSD17B2 mRNA (Fig. 2A and B).

3.4. Effect of calcitriol on HSD17B4 and HSD17B5 mRNA expression

In LNCaP cells, 10 nM calcitriol increased significantly the expression of HSD17B4 and HSD17B5 at 48 h treatment with 1.6- and 1.7-fold, respectively (Fig. 3).

3.5. Effect of LXR agonist on HSD17B4 and HSD17B5 mRNA expression

In LNCaP cells, 10 μM TO-901317 significantly up-regulates HSD17B5 but not HSD17B4 at 24 h (Fig. 4).

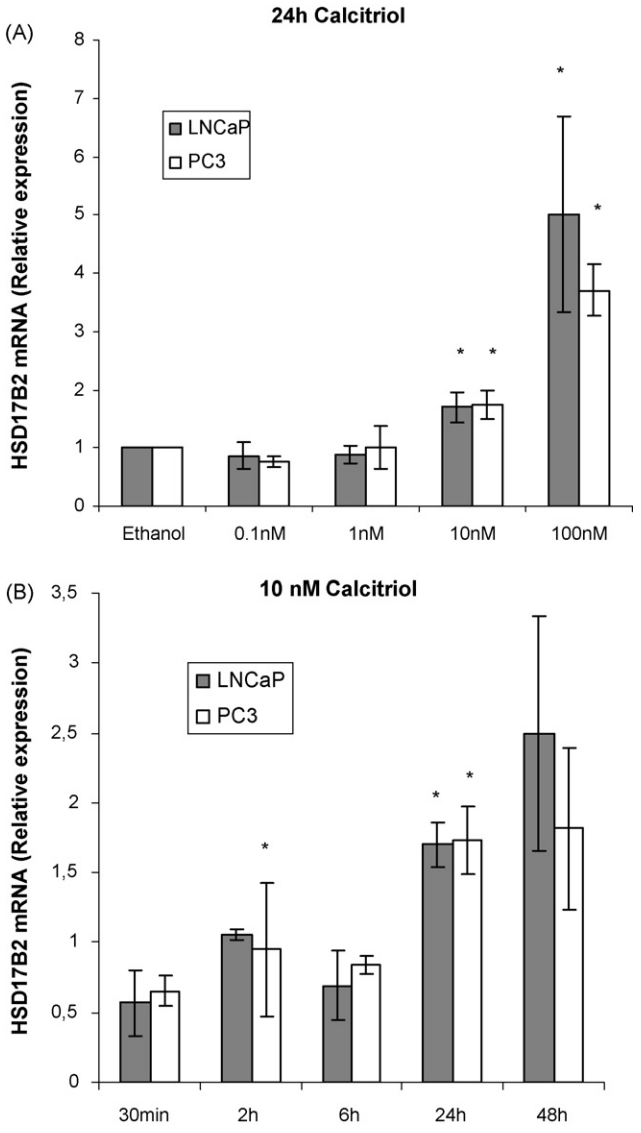

Fig. 1. Effect of calcitriol on HSD17B2 expression in LNCaP and PC3 cells. Cells were treated with different concentrations of calcitriol for 24 h (A, $^{*}P < 0.05$) or 10 nM of calcitriol for the time course indicated (B, $^{*}P < 0.05$).

3.6. Effect of DHT on HSD17B2, HSD17B4 and HSD17B5 mRNA expression

In DCC–FBS supplemented medium, 10 nM DHT up-regulates HSD17B2 mRNA to 20,000 folds at 24 h ($P = 0.003$), which is not affected by calcitriol. DHT significantly up-regulates HSD17B4 mRNA expression as well, but it significantly down-regulates HSD17B5 mRNA expression by 70% (Fig. 5). Calcitriol shows no effects on DHT mediated HSD17B4 and HSD17B5 expression (Fig. 5).

3.7. Effect of calcitriol on HSD17B2, HSD17B4 and HSD17B5 mRNA expression

In human primary prostate stromal cells, 10 nM calcitriol shows 1.4-fold ($P = 0.04$) induction of HSD17B5 expression

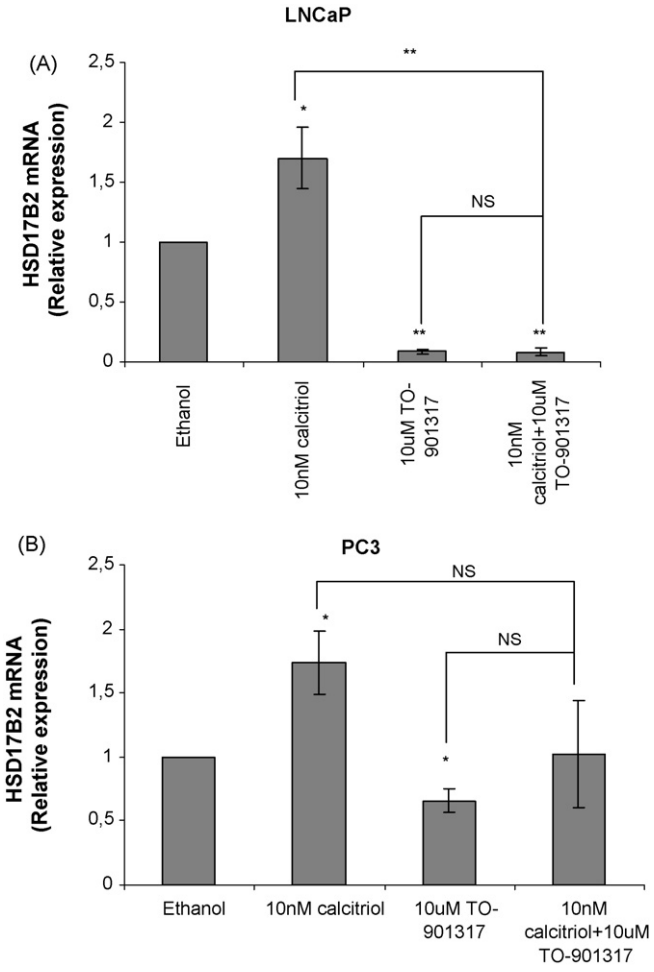

Fig. 2. Effect of TO-901317 on HSD17B2 expression in LNCaP (A) and PC3 (B) cells. Cells were treated with 10 μM TO-901317 alone or in combination with 10 nM calcitriol for 24 h ($^{*}P < 0.05$, $^{**}P < 0.001$).

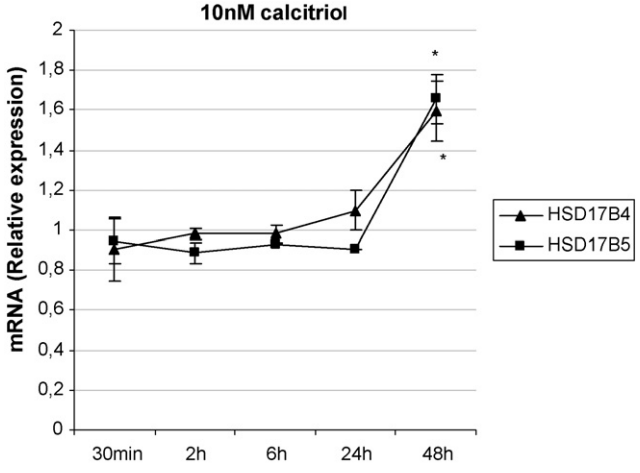

Fig. 3. Time course study of HSD17B4 and HSD17B5 expression by calcitriol in LNCaP cells. Cells were treated with 10 nM of calcitriol ($^{*}P < 0.05$).

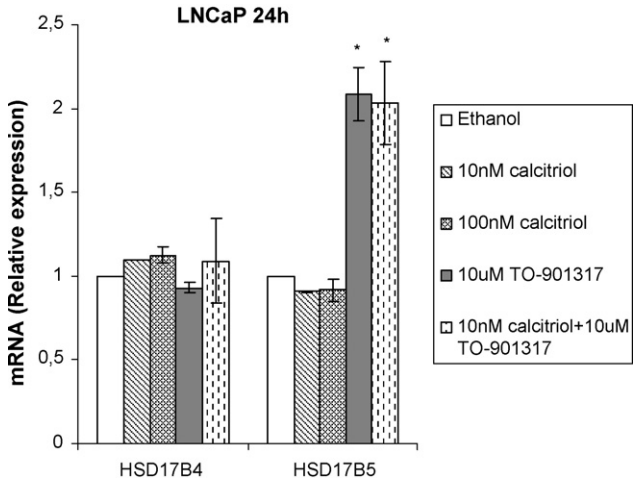

Fig. 4. Effect of TO-901317 and 100 nM calcitriol on HSD17B4 and HSD17B5 expression. LNCaP cells were treated with 100 nM calcitriol, 10 μM TO-901317 alone or in combination with 10 nM calcitriol for 24 h ($^{*}P < 0.05$).

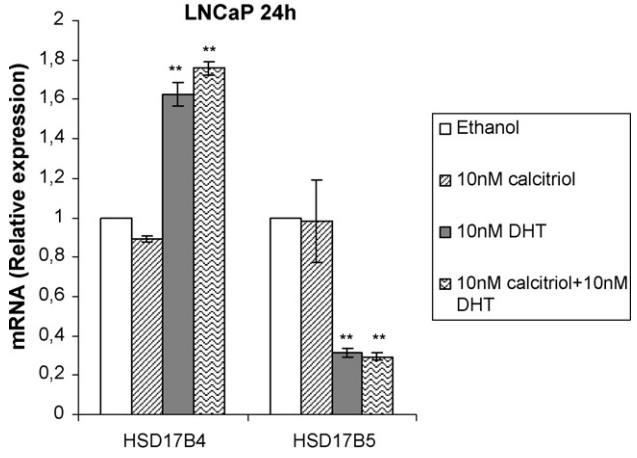

Fig. 5. Effect of DHT and calcitriol on HSD17B4 and HSD17B5 expression in DCC–FBS medium cultured LNCaP cells. Cells were treated with 10 nM calcitriol, 10 nM DHT alone or in combination for 24 h ($^{**}P < 0.001$).

at 12 h but not 30 min, 4 h, 24 h and 48 h. Calcitriol shows no effect on HSD17B2 and HSD17B5 expression at any time points above.

4. Discussion

Our results show that calcitriol up-regulates HSD17B2 in prostate epithelial cells but not in the stromal cells. However, this was observed only in the medium with serum not treated with charcoal. When the steroids of the serum were depleted by charcoal, which means low steroid but not steroid free, calcitriol lost its activity. It is possible that the action of vitamin D is androgen dependent as suggested earlier [27]. Our studies also show that calcitriol up-regulates HSD17B4, an enzyme responsible for inactivating androgen, and HSD17B5 which was reported to prefer DHT and androstenedione as

substrates and thus favor the inactivation in the prostate [17]. There are two earlier studies concerning the up-regulation of HSD17B2 by using keratinocytes [9] and using squamous carcinoma cells [28]. Our results on prostate epithelial cells are in agreement with their results. Thus, it seems that the effect of vitamin D on HSD17B2 is not cell specific.

There is an earlier study by Castagnetta et al. [22] who examined the basal level expression of HSD17B1, 2, 3, and 4 in human prostate cancer cells, but HSD17B5 was not studied. Our study shows that HSD17B2, HSD17B4 and HSD17B5 are variably expressed in LNCaP, DU145 and PC3 cells. In DU145 and PC3 cells, the expression of HSD17B4 and HSD17B5 are similar. HSD17B2 is almost undetectable in DU145 and LNCaP cells. HSD17B4 mRNA is highly expressed in LNCaP cells, where HSD17B5 expression level is low. It seems that during the transition from androgen-dependent to androgen-independent stage, the expression pattern of the three types of enzymes changes. Androgen-dependent LNCaP cells have very different expression profile compared to androgen-independent PC3 cells whereas DU145 cells are in-between. Our results show that PC3 cells have the highest expression of HSD17B2 mRNA. This expression pattern is in line with previous studies using the same cell lines [22,29]. However, they did not analyze the expression of HSD17B5. Another study carried out by Vihko and coworkers [24] reported that during the cellular transformation of LNCaP prostate cancer cells from the androgen-dependent stage to the androgen-independent stage the relative expression of HSD17B2 decreased markedly. The discrepancy may be due to the fact that PC3, LNCaP and DU145 are derived from different parental cells.

For the first time, we demonstrate that androgen up-regulates HSD17B2 mRNA expression and it is independent of which housekeeping gene is used for normalization. Because the high activity of HSD17B2 can decrease the active forms of androgens, this might imply a feed-back loop existing in the prostate for local control of androgen level. Furthermore, we also find that DHT significantly up-regulates another androgen inactivating enzyme, HSD17B4. In contrast, DHT significantly inhibits HSD17B5 expression by 30%. In the prostate, HSD17B5 enzyme seems to prefer DHT and androstenedione as substrates and favor the inactivation of androgen [17]. Taken together, the final output on androgen level is thus a complex event, but the androgen effect on HSD17B2 might be the most significant.

It is interesting that LXR agonist can inhibit HSD17B2 and induce HSD17B5 expression. Furthermore, it is able to block completely the effect of calcitriol on HSD17B2 induction. Therefore, it is possible that the effect of calcitriol on sex steroid hormone level in prostate cancer can be modified by LXR. There are few studies concerning the relationship between LXR and sex steroid hormone. Recently, it was reported that androgen concentrations were found to be reduced in 5-month-old animals null for LXRα and LXRß [25]. In a separate study, intratesticular concentrations of testosterone, progesterone, and androstenedione in the $LXR^{-/-}$ testis at 8 months were five folds lower than wild-type and the morphology of testis is altered in mutant mice [30].

In conclusion, calcitriol, LXR agonist and DHT can regulate the mRNA expression of enzymes involved in sex steroid production in prostate cancer cells and thus VDR, LXR and AR may regulate androgen and estrogen action in prostate cancer.

Acknowledgements

We are grateful to Dr. Heimo Syvälä for valuable discussion and suggestions, to Mrs. Arja Ahola, Miss Marianne Kuuslahti, Mrs. Hilkka Mäkinen and Mrs. Mirja Hyppönen for their excellent technical assistance, to Leo Pharmaceuticals (Ballerup, Denmark) for the generous gift of $1\alpha,25(OH)_2D_3$. This work was supported by grants from Tampere University Hospital, Drug Discovery Graduate School (University of Turku), the Academy of Finland and the Finnish Cancer Foundation.

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Calcitriol and TO-901317 Interact in Human Prostate Cancer LNCaP Cells

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Abstract: Vitamin D receptor (VDR) and liver X receptor (LXR) are nuclear receptors, which regulate gene transcription upon binding of their specific ligands. VDR seems to play a role in the regulation of prostate cancer cell proliferation. ATP-binding cassette transporter A1 (ABCA1) is known to be a target gene of LXR and it has been reported to be inhibited by androgen and to be involved in the regulation of LNCaP proliferation. We find that calcitriol ($1\alpha,25(OH)_2D_3$) inhibits both basal and a LXR agonist, TO-901317, induced ABCA1 mRNA expression but has no effect on the mRNA expression of ATP-binding cassette transporter G1 (ABCG1), LXRα nor LXRβ. TO-901317 increases both basal and calcitriol induced 25-hydroxyvitamin D_3-24-hydroxylase (CYP24) mRNA expression and it slightly but significantly inhibits VDR mRNA expression. The inhibition of ABCA1 by calcitriol appears to be androgen-independent. Cell growth assay shows that when each of calcitriol and 5α-dihydrotestosterone (DHT) was co-treated with ABCA1 blocker, glybenclamide, cell-growth is significantly decreased compared to their own treatments respectively. Our study suggests a possible interaction between calcitriol and TO-901317 in LNCaP cells. Alike DHT, the inhibition of ABCA1 by calcitriol may be involved in its regulation of LNCaP growth.

Abbreviations: ABCA1/G1: ATP-binding cassette transporter A1/G1; LXR: Liver X receptor; VDR: vitamin D receptor; CYP24: 25-hydroxyvitamin D_3-24-hydroxylase.

Keywords: mRNA regulation, ABCA1, CYP24, calcitriol, LXR agonist, interaction, cell proliferation

Introduction

Vitamin D receptor (VDR) is a ligand dependent transcription factor that belongs to a nuclear receptor family. Calcitriol ($1\alpha,25(OH)_2D_3$) is an active form of vitamin D_3, which mediates its biological activities through VDR. 25-hydroxyvitamin D_3-24-hydroxylase (CYP 24) is the most sensitive vitamin D_3 responsive gene (Wang et al. 2005; Wood et al. 2004) and thus its fluctuate expression after VDR activation has been utilized for evaluation of VDR signaling changes (Dunlop et al. 2005). It has been reported that calcitriol inhibits prostate cancer growth by androgen-dependent and androgen-independent mechanisms (Zhao et al. 2000).

Liver X receptors, LXRα and LXRβ are also ligand-dependent transcription factors and belong to the nuclear receptor family. Many of the target genes for LXRs are involved in cholesterol and fatty acid metabolism pathways. Major cholesterol-related targets of LXRs include the ATP-binding cassette transporter family members such as ABCA1, ABCG1, ABCG5 and ABCG8. ABCA1 is encoded by the gene that is mutated in Tangier disease (Brooks-Wilson et al. 1999; Rust et al. 1999), which is featured by low or absence of HDL-C and reduced total cholesterol (Serfaty-Lacrosniere et al. 1994) and is associated with increased susceptibility to atherosclerosis (Maxfield and Tabas, 2005). Interestingly, recent studies show that LXR agonist inhibits tumor growth and progression of LNCaP prostate cancer cells (Chuu et al. 2006; Fukuchi et al. 2004b) and androgenic inhibition of ABCA1 is involved in the regulation of prostate cancer growth (Fukuchi et al. 2004a).

Our previous study (Wang and Tuohimaa, 2007) suggests that LXR, VDR and androgen receptor (AR) signaling form a complex interaction in the prostate cancer LNCaP cells. In the present study, we aimed to investigate whether VDR ligand, calcitriol, had effects on the expression of LXR target gene,

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ABCA1, and if any, the physiological consequences concerning LNCaP cell growth; whether LXR agonist, TO-901317, had effects on the expression of VDR target gene, CYP24.

Materials and Methods

Reagents

1α,25$(OH)_2D_3$, was obtained from Leo Pharmaceuticals (Ballerup, Denmark). TO-901317, glybenclamide, cycloheximide (CHX) and RPMI-1640 medium were purchased from Sigma-Aldrich (Saint Louis, Missouri, U.S.A.). 5α-dihydrotestosterone (DHT) was obtained from Merck (Darmstadt, Germany). Bicalutamide was obtained from AstraZeneca (London, U.K.). FBS was purchased from Gibco-BRL (Life Technology, Paisley, Scotland). TRIzoL reagent was purchased from Invitrogen (Carlshad, U.S.A.). High Capacity DNA Archive Kit and SYBR Green PCR Master Mix Kit were purchased from Applied Biosystems (Forster City, U.S.A.).

Cell treatment and RNA isolation

Human prostate cancer cell line LNCaP clone FGC (American Type Culture Collection) was maintained in phenol red RPMI-1640 medium, 5 ug/ml insulin and antibiotics (penicillin 100 units/ml, streptomycin 100 ug/ml) at 37 °C in a humid atmosphere with 5% CO_2. Serum supplemented in the medium was 10% FBS otherwise 10% DCC-FBS as indicated. Cells were grown to 50% confluence and treated with vitamin D_3 or/and other reagents. Total cellular RNA was isolated with TRIzoL reagent following the instructions from the manufacturer.

Quantitative real-time PCR analysis and primer design

Quantitative Real-time PCR was performed in ABI PRISM 7000 Detection System (Applied Biosystems, U.S.A.). cDNA was synthesised using High Capacity Archive Kit (Applied Biosystems, U.S.A.) and was used as template for Quantitative Real-time PCR using SYBR Green PCR Master Mix kit (Applied Biosystems, U.S.A.). Primers were designed using Primer Express v2.0 software (Perkin-Elmer Applied Biosystems, Foster City, C.A.). BLASTn searches were performed to ensure that the primers were gene specific. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. Nucleotide sequences for primers are as following: GAPDH (**NM_002046**) Forward: 5'-CCACATCGCTCAGACACCAT-3', Reverse: 5'-ACCAGGCGCCCAATACG-3'; ABCA1 (**NM_005502**) Forward: 5'-GAGCACCATCCGGCAGAA-3', Reverse: 5'-CTCCGCCTTCACGTGCTT-3'; ABCG1 (**NM_207630**) Forward: 5'-GCTGCTGCCGCATCTCA-3', Reverse: 5'-TTCCCTTCTGCCTTCATCCTT-3'; LXRα (**HSU22662**) Forward: 5'-CATGCCTACGTCTCCATCCA-3', Reverse: 5'-CGGAGGCTCACCAGTTTCA-3'; LXRβ (**HSU07132**) Forward: 5'-GATGTCCCAGGCACTGATGA-3', Reverse: 5'-CTGGTTCCTCTTCGGGATCTG-3'; VDR (**NM_000376**) Forward: 5'-CCTTCACCATGGACGACATG-3', Reverse: 5'-CGGCTTTGGTCACGTCACT-3'; CYP24 (**NM_000782**) Forward: 5'-GCCCAGCCGGGAACTC-3', Reverse: 5'-AAATACCACCATCTGAGGCGTATT-3'

Cell growth assay

LNCaP cells were treated with hormones and/or other reagents for 0, 4 and 7 days in CellBIND 6-well plates (Sigma-Aldrich). Glybenclamide was given at a concentration which almost completely blocked the activity of ABCA1 (Becq et al. 1997). Each treatment was repeated three times. Glybenclamide was dissolved in dimethyl sulfoxide (DMSO) and all the other reagents in absolute ethanol. Control cells were treated with 0.2% ethanol plus 0.1% DMSO. Cell growth was analysed with crystal violet staining as described earlier (Kueng et al. 1989). Briefly, cells were fixed by addition of gultaraldehyde to the cell culture with a final concentration of 1% and shaking at 200 rpm for 15 min, then washed with tap water and air-dried over night. 0.1% crystal violet solution was added to stain the fixed cells for 20 min with shaking at 200 rpm. Excess dye was removed by extensive washing with tap water. The plates were air-dried over night. 10% acetic acid was used to withdraw cell-bound dye. The optical density of extracted dye was measured in 96-well plates at 590 nm by Microplate Reader (Wallac, victor 1420 multilabel counter, Turku, Finland). Final data was presented as percentage absorbance to negative control cells of corresponding treatment period.

Statistical analysis

Data of real-time PCR are expressed as the mean values ± SD. Significance was assessed using the

Student's paired *t* test. *$p < 0.05$ was considered as significant, **$p < 0.001$ as highly significant and $p > 0.05$ as not significant (NS).

Results

Down-regulation of ABCA1 mRNA by calcitriol in LNCaP cells

To test whether calcitriol has any effect on the expression of ABCA1 mRNA, prostate cancer LNCaP cells were treated with calcitriol and the mRNA expression level was analyzed by Quantitative Real-Time PCR. The results show that the expression of ABCA1 mRNA is dose dependently inhibited by $1\alpha,25(OH)_2D_3$ (Fig. 1A). At 10 nM, a significant inhibition of ABCA1 mRNA expression begins at 6 hours. At 24 hours, the effect of 10 nM $1\alpha,25(OH)_2D_3$ is at the maximum (Fig. 1B).

Calcitriol inhibits ABCA1 mRNA induction by TO-901317

Because TO-901317 induces ABCA1 expression (Wu et al. 2004), we were interested to examine whether calcitriol had any effect on ABCA1 mRNA expression in the presence of TO-901317. Our results show that TO-901317 induces 14-fold ($p = 0.0014$) of ABCA1 mRNA expression and 10 nM calcitriol with 10 uM TO-901317 results in a 47% ($p = 0.017$) decrease of it (Fig. 1C).

Effect of cycloheximide on calcitriol mediated inhibition of ABCA1 mRNA expression

Next we studied whether the decrease of ABCA1 mRNA effect of calcitriol was a direct effect. Our data show that in the presence of protein synthesis inhibitor, cycloheximide, calcitriol fails to decrease mRNA level of ABCA1 (Fig. 1D), suggesting an indirect regulation of ABCA1 by calcitriol. In comparison, cycloheximide has no effect on TO-901317 mediated ABCA1 mRNA induction (Fig. 1D).

Effect of calcitriol and TO-901313 on LXRα, LXRβ and ABCG1 expression

Alike ABCA1, ABCG1 is also a LXR target gene. Thus, we studied whether calcitriol affected the mRNA expression of ABCG1. In addition, to test whether the regulation of ABCA1 by calcitriol is via the regulation of LXRα and LXRβ, the mRNA expression of these two genes were studied as well. At 24 hours, calcitriol has no effect on any of the three genes examined at different concentrations ranging from 0.1 nM to 100 nM (Fig. 2A). In comparison, TO-901317 significantly increases 53-fold ($p = 0.03$) of the expression of ABCG1 mRNA (Fig. 2B). In the presence of calcitriol, TO-901317 increases ABCG1 expression 45-fold ($p = 0.03$) (Fig. 2B). There is no statistically significant difference between TO-901317 treatment alone and in combination with calcitriol ($p = 0.58$), implying calcitriol has no effect on TO-901317 induced ABCG1 expression as well. Cycloheximide can not block the induction of ABCG1 by TO-901317 (Fig. 2B).

Effect of TO-901317 on CYP24 and VDR expression

Studies described above deal with the effect of VDR ligand, calcitriol, on the mRNA expression of LXRs and their target genes. Next we were interested to investigate the other way around, which was to study whether LXR agonist, TO-901317, had any effect on the mRNA expression of VDR and its target gene CYP24. Our results show TO-901317 inhibits slightly but significantly the VDR mRNA expression (Fig. 3A) and there is no statistically significant difference between the TO-901317 treatment alone or in combination with calcitriol (data not shown). TO-901317 not only increases basal CYP24 mRNA expression 8-fold ($p = 0.03$) (Fig. 3A), but also enhances 10-fold ($p < 0.001$) of calcitriol mediated induction of CYP24 expression (Fig. 3B).

Androgen-dependency of ABCA1 expression

It has been reported that androgen inhibits ABCA1 expression in LNCaP cells (Fukuchi et al. 2004a). Actions of vitamin D involve both androgen-dependent and androgen-independent mechanisms (Zhao et al. 1997; Zhao et al. 1999; Zhao et al. 2000). Thus we examined whether the inhibition of ABCA1 mRNA expression was androgen-dependent. As a positive control, androgen was included in our study. We also find that androgen inhibits the expression of ABCA1 (Fig. 4B). In the

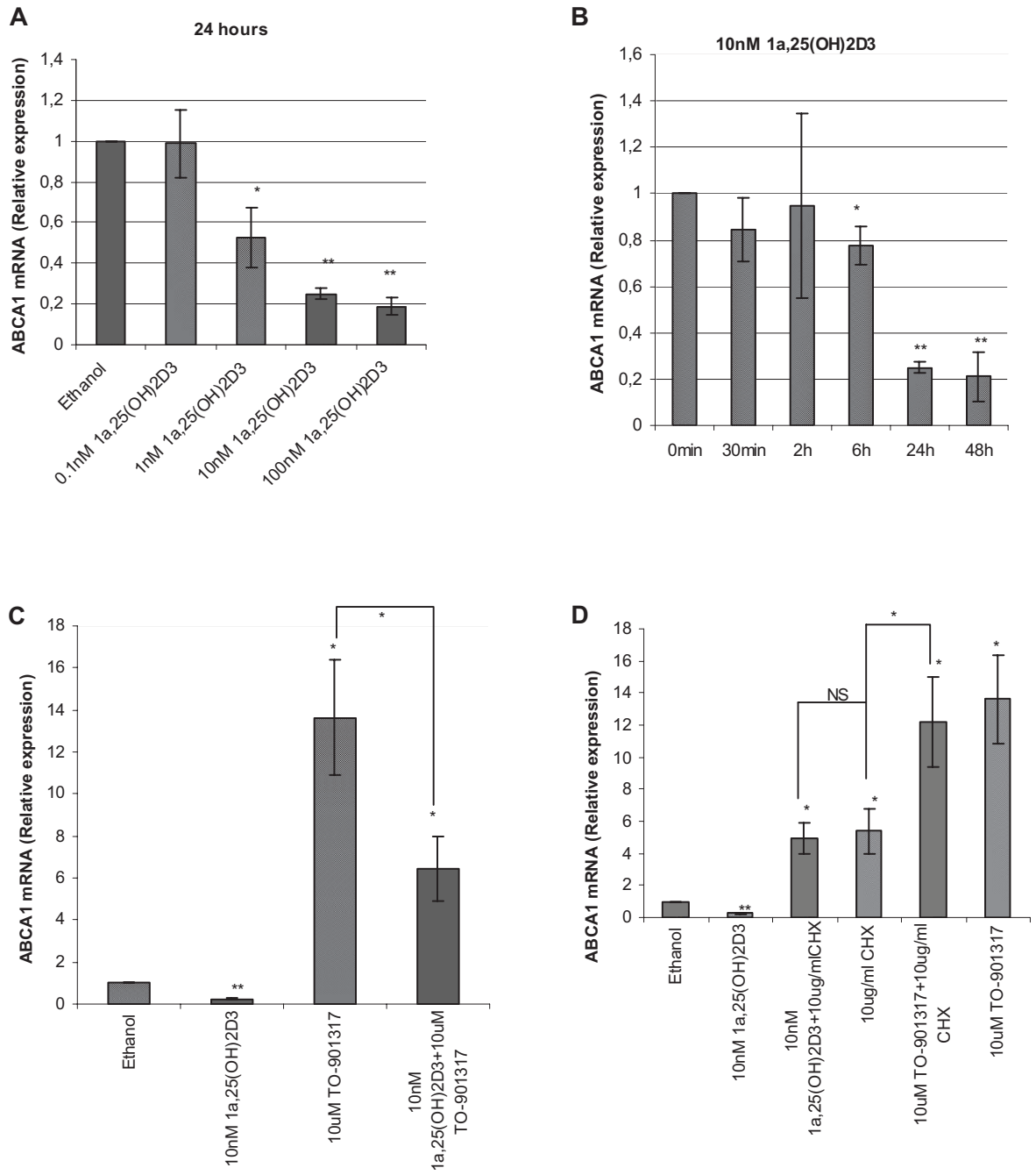

Figure 1. Down-regulation of ABCA1 mRNA expression by calcitriol in LNCaP cells. (**A**) LNCaP cells were treated with 0.2% ethanol and different doses of calcitriol for 24 hours or (**B**) with 0.2% ethanol and 10 nM calcitriol for different period of time. (**C**) Cells were treated with 0.2% ethanol, 10 nM $1\alpha,25(OH)_2D_3$, 10 uM TO-901317 and $1\alpha,25(OH)_2D_3$ plus TO-901317. The inhibition of ABCA1 occurred also in the presence of TO-901317. (**D**) Cells were treated with 0.2% ethanol, 10 nM $1\alpha,25(OH)_2D_3$, 10 ug/ml cycloheximide, 10 uM TO-901317 and $1\alpha,25(OH)_2D_3$/ TO-901317 plus cycloheximide. Cycloheximide has no effect on TO-901317 mediated ABCA1 mRNA induction but blocked the inhibition effect of calcitriol. Relative ABCA1 mRNA expression was analysed by Quantitative Real-Time PCR ($n = 3$, $^*p < 0.05$, $^{**}p < 0.001$).

medium supplemented with the normal serum, androgen receptor antagonist, bicalutamid, causes a significant increase of ABCA1 expression (Fig. 4A). Despite the presence of bicalutamide, calcitriol decreases significantly the mRNA expression level of ABCA1 to the level with calcitriol treatment alone (Fig. 3A). Furthermore, in medium containing dextran-charcoal-stripped serum (low androgen serum), which is referred to DCC medium herein, 10 nM calcitriol still significantly decreases ABCA1 expression by 64% ($p < 0.001$) (Fig. 4B).

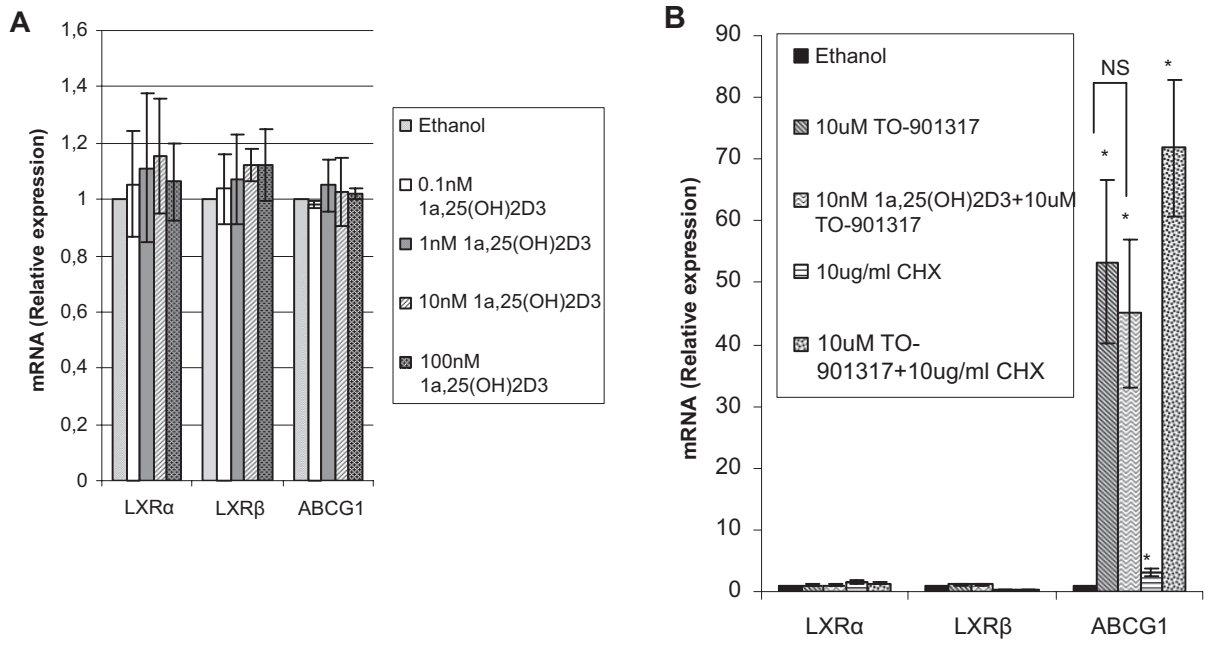

Figure 2. Effects on the mRNA expression of LXRα, LXRβ and ABCG1 by calcitriol, TO-901317 and cycloheximide. LNCaP cells were treated for 24 hours with 0.2% ethanol, and (**A**) different concentrations of calcitriol (**B**) 10 uM TO-901317, 10 ug/ml cycloheximide, TO-901317 plus calcitriol and/or plus cycloheximide. Calcitriol has no effect on the mRNA expression of LXRα, LXRβ and ABCG1 whether TO-901317 is present (**B**) or not (**A**). Relative mRNA expression were analysed by Quantitative Real-Time PCR (n = 3, *$p < 0.05$, **$p < 0.001$).

Effect of calcitriol, TO-901317, DHT and glybenclamide on LNCaP proliferation

Finally, we were interested to study whether the inhibition of ABCA1 by calcitriol rendered any physiological effect regarding LNCaP growth, and whether calcitriol and DHT had the same effect on LNCaP cell proliferation when they were individually co-treated with glybenclamide or TO-901317. Figure 5 shows that day4 and day7 treatments have the same trend for each treatment indicated. In comparison to the negative control,

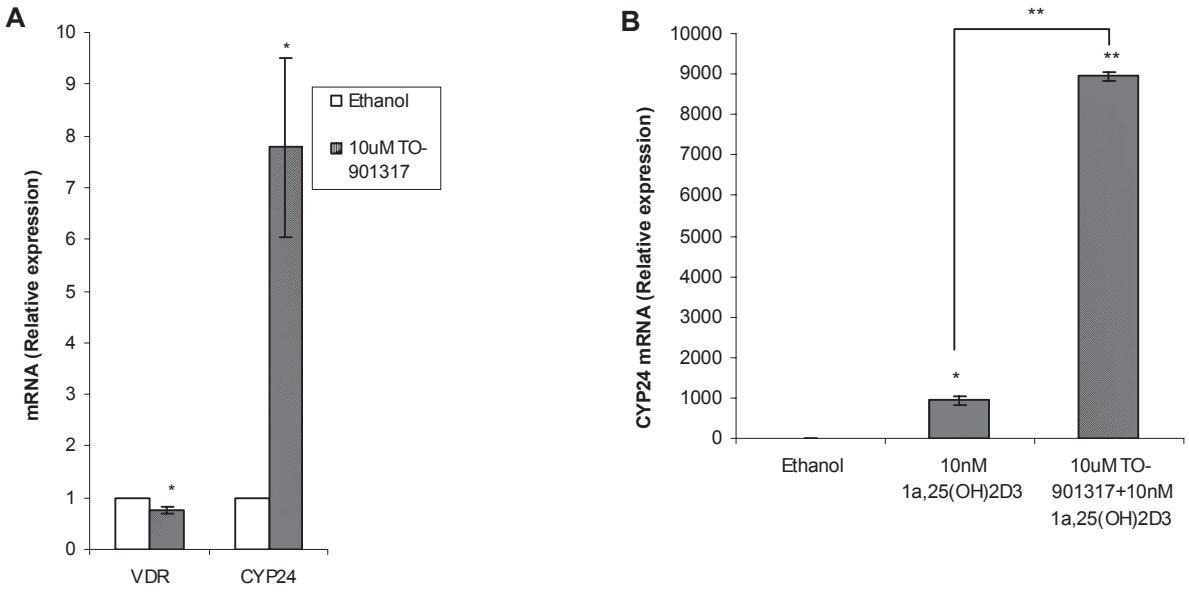

Figure 3. Effects on the mRNA expression of CYP24 and VDR by TO-901317. LNCaP cells were treated with 0.2% ethanol, 10 nM calcitriol and 10 uM TO-901317 alone or in combination for 24 hours. TO-901317 inhibits VDR but induces basal CYP24 expression (**A**) as well as calcitriol mediated CYP24 induction (**B**). Relative mRNA expression were analysed by Quantitative Real-Time PCR (n = 3, *$p < 0.05$, **$p < 0.001$).

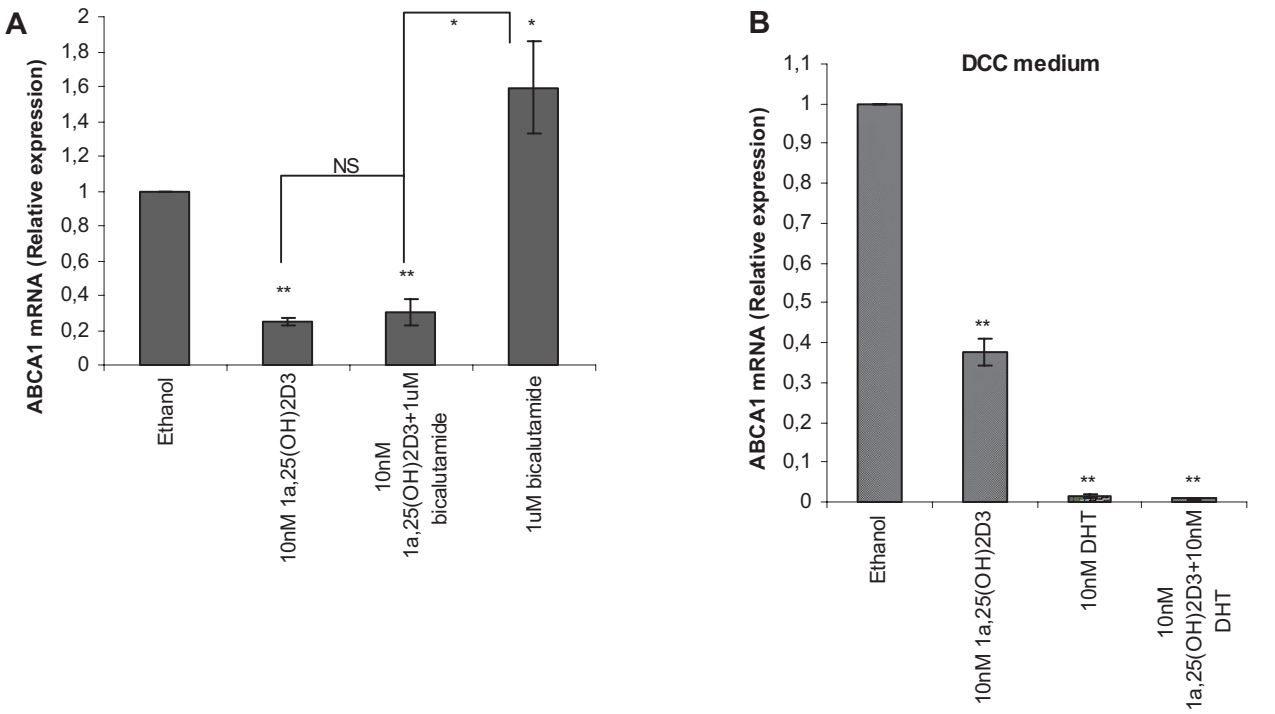

Figure 4. Androgen-dependency of ABCA1 expression. LNCaP cells were treated for 24 hours with 0.2% ethanol, 10 nM calcitriol, 1 uM bicalutamide and calcitriol plus bicalutamide in normal medium (**A**), or with 10 nM calcitriol, 10 nM DHT and calcitriol plus DHT in DCC medium (**B**). Relative mRNA expression was analyzed by Quantitative Real-Time PCR ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.001$).

which is 100% in relative absorbance, both calcitriol and DHT decreases LNCaP cell growth. At day7, when each of calcitriol and DHT is co-treated with glybenclamide, cell growth is significantly decreased compared to their own treatment respectively. However, when co-treated with TO-901317, calcitriol shows no significant difference and DHT shows significant decrease of cell proliferation compared with each hormone treatment alone, respectively. TO-901317 and glybenclamide individually decreases LNCaP cell growth. Their co-treatment further decreases cell proliferation, which remains unchanged whether when calcitriol or DHT is added.

Discussion

In this study, we report for the first time, that calcitriol inhibits the mRNA expression of ABCA1. Calcitriol not only decreases basal level of ABCA1 expression, but also inhibits the LXR agonist, TO-901317, mediated induction of ABCA1. On the other hand, TO-901317 alone increases CYP24 mRNA expression and, furthermore, it enhances calcitriol mediated induction of this gene. The increase of CYP24 mRNA by TO-901317 is not due to an increase of VDR. Calcitriol has no effects on LXRα and LXRβ expression, suggesting that the inhibition of ABCA1 by calcitriol is not due to the down-regulation of these two transcription factors. Previously, Takahide M et al. (Jiang et al. 2006) reported that $1\alpha,25(OH)_2D_3$ blunted the LXRα-mediated induction of cholesterol 7alpha-hydroxylase mRNA in H4IIE rat hepatoma cells. Cholesterol 7alpha-hydroxylase is the rate-limiting enzyme in the catabolism of cholesterol to bile acid (Hubacek and Bobkova, 2006; Shefer et al. 1970) and it is stimulated by oxysterol receptor, LXRα (Chiang et al. 2001; Peet et al. 1998). Thus, it appears that VDR negatively regulates LXR mediated induction of ABCA1 as well as cholesterol 7alpha-hydroxylase, which are all involved in cholesterol efflux and bile acid synthesis. On the other hand, we find that TO-901317 slightly but significantly inhibits VDR expression and up-regulates CYP24, which is up-regulated by VDR (Chen and DeLuca, 1995). CYP24 is an enzyme responsible for inactivating of active vitamin D metabolites such as $1,25\alpha(OH)_2D_3$ and $25(OH)D_3$ (Chen et al. 1993;

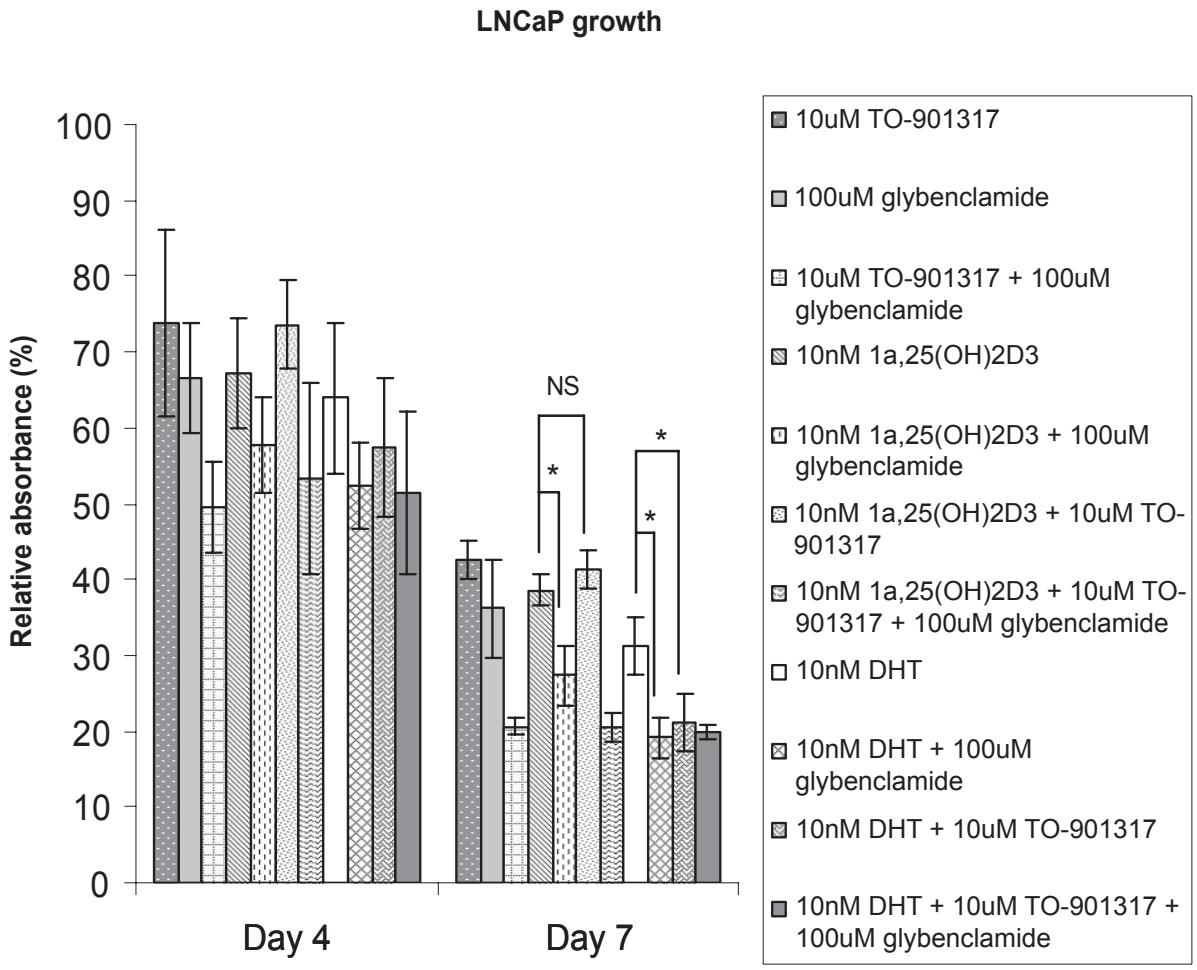

Figure 5. Effect of calcitriol, TO-901317, DHT and glybenclamide on LNCaP proliferation. LNCaP cells were treated for 0, 4 and 7 days (data at Day 4 and 7 are given) with 1.10 uM TO-901317; 2.100 uM glybenclamide; 3.10 uM TO-901317+100 uM glybenclamide; 4.10 nM 1α,25$(OH)_2D_3$; 5.10 nM 1α,25$(OH)_2D_3$+100 uM glybenclamide; 6.10 nM 1α,25$(OH)_2D_3$ +10 uM TO-901317; 7.10 nM 1α,25$(OH)_2D_3$+10 uM TO-901317+100 uM glybenclamide; 8.10 nM DHT; 9.10 nM DHT+100 uM glybenclamide; 10.10 nM DHT+10 uM TO-901317; 11.10 nM DHT+10 uM TO-901317+100 uM glybenclamide. Negative control cells were treated with 0.2% ethanol + 0.1% DMSO. Crystal violet staining analysis was used to measure the absolute absorbance which reflects absolute cell number. Data shown is relative absorbance (Relative absorbance = (absolute absorbance)$_{compound}$ / (absolute absorbance)$_{ethanol}$ * 100). Day4 and day7 treatments have the same trend for each treatment indicated. ($n = 3$, *$p < 0.05$, **$p < 0.001$).

Ohyama et al. 1993). This suggests that LXR agonist might play a role in the negative regulation of the actions of VDR ligand. Stimulated by a growing body of preclinical evidence that vitamin D inhibits the proliferation of prostate cancer, clinical trials of calcitriol in prostate cancer patients have been carried out since the 1990s (Beer, 2003). On the other hand, our present study shows that TO-901317 inhibited LNCaP cell proliferation and Liao et al. (Fukuchi et al. 2004a) find that knockdown of ABCA1 expression by RNA interference in androgen-dependent cells increased their rate of proliferation. Based on their study they proposed a potential of use of LXR signaling as a therapeutic target in prostate and other cancers (Chuu et al. 2007). Our present study suggests a mutual negative regulation of the actions of the ligands of VDR and LXR. This implies that cautions have to be taken whether which of the VDR or LXR signaling was considered for the therapeutic target in prostate cancer.

ABCA1 has been reported to be regulated by androgen in LNCaP cells (Fukuchi et al. 2004a). Our study shows in normal medium, AR antagonist, bicalutamide, increases ABCA1 level and calcitriol acts as an antagonist of bicalutamide. Furthermore, calcitriol significantly inhibits ABCA1 in low-androgen-serum containing medium. This suggests that the inhibition of ABCA1 by calcitriol is most probably androgen-independent.

ABCA1 is a cholesterol exporter (Oram and Heinecke, 2005; Venkateswaran et al. 2000).

It may be involved in cell proliferation as reported earlier (Fukuchi et al. 2004a), which showed specific knock-down of ABCA1 expression by RNA interference resulted in an increase of LNCaP proliferation. The same research group (Fukuchi et al. 2004a) demonstrated that DHT inhibited ABCA1 expression and this regulation is involved in DHT mediated LNCaP proliferation. Here we find that calcitriol also inhibits the expression of ABCA1. Thus, we were interested to test whether the inhibition of ABCA1 by calcitriol had effect on cell proliferation, and we included DHT as a parallel in our cell growth assay. We did not observe an increase of cell proliferation after glybenclamide treatment as expected, given that knock-down of ABCA1 increases LNCaP proliferation (Fukuchi et al. 2004a). This might be because glybenclamide can inhibit a broad range of ABC transporters, including ABCA1 and cystic fibrosis transmembrane conductance regulator (Hasko et al. 2002). The final output of specific blocking of ABCA1 by glybenclamide thus is not seen here. This explains why co-treatment of glybenclamide and TO-901317 dose not give a cell growth level which is in between of the level from each reagent treatment alone, but it is even further decreased compared to either of the reagent treatment. Thus, here we could not draw any conclusion by comparing calcitriol treatment and calcitriol plus glybenclamide treatment. However, when each of calcitriol and DHT was co-treated with glybenclamide, cell-growth was significantly decreased compared to their own treatment respectively. This suggests that the regulation of LNCaP cell proliferation by calcitriol may involve its inhibition of ABCA1, which has some similarity to DHT, given that inhibition of ABCA1 by DHT is involved in its regulation of LNCaP proliferation (Fukuchi et al. 2004a). However, when co-treated with TO-901317, calcitriol shows no significant difference whereas DHT shows significant decrease of cell proliferation when compared with each hormone treatment alone, respectively, suggesting that DHT is more involved in LXR agonist related cell growth regulation than calcitriol does.

The inhibition of ABCA1 mRNA by calcitriol occurred not earlier than at 6 hours, indicating that the effect might be indirect. This was confirmed by using protein synthesis inhibitor, cycloheximide, which blocked calcitriol mediated decrease of ABCA1 mRNA. In comparison, it failed to block LXR agonist mediated induction of ABCA1 and ABCG1, suggesting ABCA1 and ABCG1 are directly regulated by LXR, which have been reported before (Costet et al. 2000; Karten et al. 2006). The testing of ABCA1 and ABCG1 serves as positive control in our experiments and the testing results indicate that our experimental system works normally.

In conclusion, we report for the first time that VDR ligand, calcitriol, inhibits ABCA1 mRNA expression and LXR agonist, TO-901317, induces CYP24 mRNA expression, suggesting an interaction between calcitriol and TO-901317 in prostate cancer cells, which implicates an association between VDR and LXR in prostate cancer. The inhibition of ABCA1 by calcitriol seems to be androgen-independent and might be involved in LNCaP proliferation, which has at least some similarity to DHT. Studying and understanding of the interaction between VDR and LXR/AR is critical for rational design of future clinical trials with vitamin D compounds for prevention and treatment of associated cancers such as prostate cancer.

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Serum cholesterol and expression of ApoAI, LXRβ and SREBP2 in vitamin D receptor knockout mice

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Abstract

Vitamin D insufficiency has been reported to be associated with increased blood cholesterol concentrations. Here we used two strains of VDR knockout (VDR-KO) mice to study whether a lack of vitamin D action has any effect on cholesterol metabolisms. In 129S1 mice, both in male and female VDR-KO mice serum total cholesterol levels were significantly higher than those in wild type (WT) mice (20.7% (P= 0.05) and 22.2% (P=0.03), respectively). In addition, the serum high-density lipoprotein-bound cholesterol (HDL-C) level was 22% (P=0.03) higher in male VDR-KO mice than in WT mice. The mRNA expression levels of five cholesterol metabolism related genes in livers of 129S1 mice were studied by using quantitative real-time PCR (QRT-PCR): ATP-binding cassette transporter A1 (ABCA1), regulatory element binding protein (SREBP-2), apolipoprotein A-I (ApoAI), low density lipoprotein receptor (LDLR) and liver X receptor beta (LXRβ). In the mutant male mice, the mRNA level of ApoAI and LXRβ were 49.2% (P=0.005) and 38.8% (P=0.034) higher than in the WT mice. These changes were not observed in mutant female mice, but the female mutant mice showed 52.5% (P=0.006) decrease of SREBP2 mRNA expression compared to WT mice. Because the mutant mice were fed with a special rescue diet, we wanted to test, whether the increased cholesterol levels in mutant mice were due to the food. Both the WT and mutant NMRI mice were given the same food for 3 weeks before the blood sampling. No difference in cholesterol or in HDL-C between WT and mutant mice was found. The results suggest

that the food, gender and genetic background have an effect on the cholesterol metabolism. Although VDR seems to regulate some of the genes involved in cholesterol metabolism, its role in the regulation of serum cholesterol seems to be minimal.

Key words: vitamin D receptor; cholesterol; mice; high-density lipoprotein-bound cholesterol; gene expression

1. Introduction

Vitamin D is produced in the skin through a photolytic reaction of 7-dehydrocholesterol induced by ultraviolet B radiation at 290-315 nm. The vitamin D formed in the skin or absorbed from diet are hydroxylated in the liver to 25-hydroxyvitamin D and further hydroxylated in the kidney to 1,25-dihydroxyvitamin D (calcitriol) [1]. Calcitriol is the most active ligand for vitamin D receptor (VDR) and after binding to VDR performs its biological functions such as control of calcium homeostasis, cell proliferation and differentiation [2, 3]. Lack of sunlight and the vitamin D deficiency has been suggested to be associated with an increased blood cholesterol concentration [4]. In postmenopausal women with hormone replacement therapy, vitamin D seems to affect serum lipid levels [5]. Therefore we were interested to study the effect of VDR knock out in mice on their serum cholesterol level.

SREBP2 is a transcription factor which down-regulates ABCA1 [6] but it increases LDLR expression [7] and is involved in cholesterol synthesis [8]. Many of the LXR target genes are also involved in cholesterol and fatty acid metabolism pathways [9-11].

Thus, LXRs form heterodimer with RXR and act as cholesterol sensors as well as regulators of genes for cholesterol efflux and lipid transport to maintain cholesterol homeostasis [12-15]. Major cholesterol-related targets of LXRs include the ATP-binding cassette transporter family members such as ABCA1 [16, 17], which is mutated in Tangier disease. A characteristic feature of these patients is extremely low or absent HDL-C and reduced total cholesterol [18-20]. The main role of ABCA1 is the mediation of the efflux of excess cholesterol from macrophages and fibroblasts in the sub-endothelial space to acceptors such as HDL and ApoAI [21], which in the circulation interacts first with serum phospholipids and forms nascent discoidal HDL (ndHDL), which triggers cholesterol efflux [21]. The externalized cholesterol is incorporated into ndHDL and further modified. The final products, cholesteryl esters, are delivered back to the liver via LDLR, converted to bile salts, and eliminated through the gastrointestinal tract [21]. LDLR knockout mice showed altered lipid profile [22]. Therefore, in the present study, we tested the mRNA expression of ABCA1, ApoAI, LDLR, LXRβ and SREBP2 from both wild type and mutant mice.

2. Materials and methods

2.1. Mouse breeding, housing and feeding

VDR-KO mice 129S1 were produced from the line initially generated in the University of Tokyo [23], which have been studied in our laboratory [24-26]. NMRI mice were purchased from Harlan, Nederland. They originate from Swiss mice in the US brought from Lausanne, Switzerland, in 1926 by Clara Lynch. In 1937 the mice came from Lynch to Poiley and were inbred by Poiley known as NIH/PI. At F51 they went to US Naval Medical Research Institute and thus known as NMRI. In 1955, the mice went to Bundes-

Forschungsanstalt für Viruskrankheiten and in 1958, to Central Institute for Laboratory Breeding, Hannover. In 1981, they came from Central Institute for Laboratory Breeding, Hannover to Winkelmann (now Harlan Winkelmann). In 1998, they came from Harlan Winkelmann to Harlan Nederland (www.harlaneurope.com), known as HsdWin:NMRI mice, which are abbreviated as "NMRI mice" in the text. Studies using this strain of mice have been reported [27, 28]. All the mice were housed in the University of Tampere Laboratory Animal facility with a 12:12-light:dark cycle. The management and experimental procedures in this study were approved by the Ethical Committee of the University of Tampere and performed according to EU legislation. Age-matched wild type and VDR-KO mice were genotyped and used in this study. Mice were 6.5-16 months old. The numbers of 129S1 mice used in the study were, male KO 8 and male WT 9; female KO 6 and female WT 5. The numbers of NMRI mice were, male KO 2 and WT 10, female KO 7 and WT 11. The VDR-KO mice were fed with a special diet containing 2% Ca, 1.25% P and 20% lactose (Lactamin AB, Sweden), to normalize their mineral metabolism. In the studies with NMRI mice, WT mice were switched from normal foods (0.9% Ca, 0.7% P and 20% lactose) to special foods 3 weeks before sample collection.

2.2. Serum sample preparation and tissue sample collection

Mice were sacrificed by carbon dioxide and blood was immediately taken by heart puncture. Blood was allowed to clot, followed by centrifugation at 3000rpm for 10min. The serum was stored at -70ºC for further analysis. Small pieces of liver and kidney tissues were taken and dropped into RNAlater (Ambion), and stored at -20ºC for later RNA isolation.

2.3. Measurement of total cholesterol and HDL-C

Total cholesterol and HDL-C concentrations were measured using a photometric CHOD-PAP (Ecoline® S+ Cholesterol, DiaSys Diagnostic Systems GmbH, Germany). HDL-C was determined from the clear supernatant after precipitation of serum apoB-containing lipoproteins with 10% polyethylene glycol (final concentration).

2.4. RNA extraction and real-time quantitative PCR

Liver tissue slices was dropped into ice-cooled trizol (Invitrogen, Carlshad, USA) and homogenized on ice. Total cellular RNA was isolated with TRIzoL reagent following the instructions from the manufacturer. The RNA concentration was calculated from absorbance at 260nm in a GeneQuant II (Pharmacia Biotech, USA) and A280/A260 was measured to verify the purity of the RNA. The ratio of all the RNA samples fell in 1.9-2.1. Randomly selected RNA samples were subjected to denaturing-gel electrophoresis. The ratio of the intensity of the 28S band and that of the18S band was 1.5-2.0. cDNA was synthesised by using High Capacity Archive Kit (Applied Biosystems, USA). Real-time PCR were performed in ABI PRISM 7000 Detection System (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Applied Biosystems). The programs for the amplification were as following: activation of polymerase at 95ºC for 10min, followed by 45 cycles of denaturation at 95ºC for 15sec and annealing/extension at 60ºC for 1min. The analysis of dissociation curves was always performed after 45 cycles. Primers were designed by using Primer Express v2.0 software (Perkin-Elmer Applied Biosystems, Foster City, CA). BLASTn searches were performed to ensure that the primers were gene specific. The primers are mABCA1 (**NM_013454**) forward 5'-CAACCCCTGCTTCCGTTATC-3', reverse 5'-GACCTTGTGCATGTCCTTAATGC-

3’; mApoAI **(NM_009692)** forward 5’- CTCCTCCTTGGGCCAACA-3’, reverse 5’-TGACTAACGGTTGAACCCAGAGT-3’; mLDLR (**NM_010700**) forward 5’-TGTGAAAATGACTCAGACGAACAA-3’, reverse

5’- GGAGATGCACTTGCCATCCT-3’; mLXRβ (Nr1h2) (**NM_009473**) forward 5’-GATCCTCCTCCAGGCTCTGAA-3’, reverse 5’-TGCGCTCAGGCTCATCCT-3’; mSREBP2 (**NM_033218**) forward 5’-GTGCGCTCTCGTTTTACTGAAGT-3’, reverse 5’- GTATAGAAGACGGCCTTCACCAA-3’. Mouse gene β-ACTIN (**NM_007393**) was used as endogenous control. Primers for mβ-ACTIN were as follows: forward: 5’-GCTTCTTTGCAGCTCCTTCGT-3’ reverse: 5’-CCAGCGCAGCGATATCG-3’.

2.5. Statistical analysis

Data of cholesterol and HDL-C level as well as real-time PCR are expressed as the mean value ± standard error. Significance was assessed using the Mann-Whitney *U* test. *$p \leq 0.05$ was considered as significant, **$p < 0.001$ as highly significant and $p > 0.05$ as not significant (NS).

3. Results

3.1. Total cholesterol and HDL-C serum concentration

The mean total cholesterol concentration was significantly higher in 5 female mutant mice in comparison with that of 5 female wild type mice (Table1). In female mice, there was no statistically significant difference between in the serum HDL-C. In male mice, both total cholesterol and HDL-C concentrations were significantly lower in WT mice than in KO mice (Table 1).

3.2. ABCA1, ApoAI, LDLR, LXRβ and SREBP2 mRNA expression in the liver

QRT-PCR data show a significant difference in ApoAI, LXRβ and SREBP2 expressions between WT and mutants (Figure 1A and 1B). The relative mRNA levels were 1.0 ± 0.2 and 0.7 ± 0.1 (P=0.005) for ApoAI, 1.1 ± 0.3 and 0.8 ± 0.2 (P=0.034) for LXRβ in mutant and wild type male mice, respectively; 0.9 ± 0.1 vs 1.4 ± 0.3 (P=0.006) for SREBP2 in mutant and wild type female mice, respectively. The female wild type and mutant mice don't show difference in the expression of ApoAI nor LXRβ. There is no significant difference of the expression of ABCA1 and LDLR in mutant and wild type mice.

3.3. The effect of food

We used NMRI strain of VDR-KO mice. Both the WT and mutant mice were given the same foods containing 2% Ca, 1.25% P and 20% lactose for 3 weeks before blood sampling. No difference in cholesterol or in HDL-C between WT and mutant mice was found, but a clear sexual difference was found (Table 2).

4. Discussion

This is the first direct evidence that a nonfunctional VDR can increase serum total cholesterol concentration in both female and male mice. Also HDL-C was increased but only in the male VDR-KO mice. In male animals, the higher total cholesterol level in KO mice was due to increase in both HDL-C and apoB-containing lipoprotein cholesterol. It seems that the mutation of VDR in male and female mice have different effects on lipids, which is consisted with relative gene expression changes. In male lacking of functional VDR, ApoAI and LXRβ expression levels were increased, but not in females. In VDR-KO females, expression of SREBP2 was decreased. It has been reported that calcitriol inhibits ApoAI mRNA and protein in the human hepatoma cell line HepG2 VDR- and

VDRE-dependently [29]. In our study, male mutant mice had a higher liver ApoAI mRNA expression. Thus, it is possible that VDR knockout results in a higher ApoAI level, which in turn, increased cholesterol efflux and hence HDL-C.

In mammals there are two forms of LXRs, LXRα/NR1H3 and LXRβ/NR1H2. The expression of LXRα is restricted to kidney, intestine, spleen, and adrenals, with the highest expression levels in the liver [30, 31] whereas the LXRβ is ubiquitously expressed [32, 33]. Inhibition of LXRα signaling by vitamin D receptor has been reported [34]. Because of the high expression of LXRα in the liver, its role in lipid metabolism has been extensively studied. A search in PUBMED with "LXRβ + lipid" and "LXRα + lipid" revealed 125 and 792 articles, respectively. Thus, in the present study we investigated the LXRβ expression. Interestingly, male mutant mice have higher levels of LXRβ mRNA expression. As one of the target genes for LXRβ, it has been shown that an over-expression of ABCA1 in liver is associated with increased HDL-C levels in transgenic mice and ABCA1 knockout leads to HDL-C deficiency [35]. Our quantitative real-time PCR results show that the liver ABCA1 mRNA expression levels are unchanged. This suggests that other factors than ABCA1 are involved in the serum lipid changes of the mutant mice. Given the role of LXRβ for activating not only ABCA1, but also ABCG1 [36], ABCG5 [37] and ABCG8 [38] it is possible that the LXRβ exerts its effects on HDL-C levels by activating other cholesterol exporters. In addition, vitamin D inhibits LXRα signaling [34], which thus in VDR-KO mice might be enhanced and consequently contributes to increase ABCG1, ABCG5 and ABCG8 expression as well.

SREBPs are synthesized as precursors complexed with SCAP in the membranes of the endoplasmic reticulum (ER) [39]. When cells are depleted of sterols, SCAP escorts SREBPs from ER to Golgi where the SREBPs are cleaved [40, 41] to release the bHLH-Zip domain which travels to the nucleus where it activates genes whose products play roles in the lipid synthesis and uptake [42], including cholesterol [43]. Our results showed that VDR mutant female mice have lower levels of SREBP2 but higher level of cholesterol. Cholesterol has been shown directly bind to sterol-sensing domain of SCAP *in vitro* [44]. The binding of cholesterol to SCAP elicits a conformational change in SCAP, which causes SCAP to bind to Insigs, which are endoplasmic reticulum retention proteins that abrogate movement of the SCAP-SREBP complexes to the Golgi apparatus for SREBP processing and activation [44-47]. It has been reported murine Insig-2 promoter harbors a positive vitamin D response element [48]. SREBP2 is a transcription factor which inhibits ABCA1 [6] but stimulate LDLR [7] expression. Mutant female mice showed lower SREBP2 expression but no changes in ABCA1 nor LDLR were observed.

It seems that the special rescue diet of the mutant mice has a strong effect on the serum lipid levels. In our experiments using NMRI mice, both wild type and mutant mice were fed with special food containing 2% Ca, 1.25% P and 20% lactose three weeks before the sampling. No significant difference in cholesterol and/or HDL-C level between wild type and mutant mice was found. Because there were only 2 NMRI male VRD-KO mice available for the studies, it remains unclear whether this is because of the effects of food or the strain of the mice. On the other hand, this might imply that the effect of VDR

knockout on cholesterol metabolism can be rescued by diet. Investigations of all the four VDR-KO mice models to date demonstrated that the lack of functional VDR develops hypocalcemia, rickets, osteomalacia, hyperparathyroidism and alopecia [23, 49-51]. Normalization of ionized calcium levels with the rescue diet restored all the other phenotypes except for alopecia [52, 53].

The sexual difference of cholesterol and HDL-C was found in both 129S1 and NMRI strains in wild type and mutant mice. Cholesterol changes in postmenopausal women [54] and in rodents after ovariectomy [55, 56] are due to the lack of estrogen. Kamei et al. [57] identified the changes of gene expression in lipid metabolism including a decrease of SREBP1 in ovariectomized mice. Similarly, here we found that SREBP2 level was lower in VDR-KO female mice. Previously it was reported that VDR-KO female mice developed uterine hypoplasia in the post-weaning stage due to a lack of estrogen synthesis in the mutant ovaries [23]. Our present study show that the VDR background has different effects in female and male mice, where nonfunctional VDR can increase serum total cholesterol concentration in both female and male mice but HDL-C was increased only in the male VDR-KO mice. This difference might due to the impaired estrogen production in KO female mice.

In conclusion, our study suggests that lack of the functional VDR may lead to an increased serum cholesterol and HDL-C. In addition, the changes can be partially explained by changes in the expression of cholesterol metabolism related genes such as ApoAI, LXR and SREBP2. Vitamin D deficiency may therefore contribute to

cardiovascular diseases such as atherosclerosis [58]. However, also the gender and the food have a clear effect on the serum lipid levels independently on the VDR mutation. Therefore, the role of VDR in the regulation of serum cholesterol seems to be minimal.

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Figures and Tables

Table 1.

Serum cholesterol and HDL-C level in wild type and VDR knockout 129S1 mice

Female

	WT	VDR-KO	P value
Total cholesterol	2.14 ± 0.09	2.62 ± 0.15	0.028
HDL-C	1.23 ± 0.06	1.35 ± 0.04	0.209
Nr of observation	5	5	

Male

	WT	VDR-KO	P value
Total cholesterol	2.54 ± 0.07	3.1 ± 0.27	0.054
HDL-C	1.69 ± 0.05	2.04 ± 0.16	0.034
Nr of observation	9	8	

Serum cholesterol and HDL-C levels of wild type and VDR knockout mice were determined. Data were analyzed by Mann-Whitney *U* test and presented as mean value ± standard error. The unit used in the table is mmol/L (SI Unit). To convert values from SI units to conventional units, divide by the conversion factor. For cholesterol and HDL-C, the convert factor is 0.0259.

Table 2.

Serum cholesterol and HDL-C level in wild type and VDR knockout NMRI mice

WT				
		Female	Male	P value
	Total cholesterol	3.71 ± 0.08	5.15 ± 0.09	0.00108
	HDL-C	2.76 ± 0.06	4.14 ± 0.07	0.00011
	Nr of observation	11	10	
VDR-KO				
		Female	Male	P value
	Total cholesterol	3.85 ± 0.07	5.19 ± 0.03	0.0421
	HDL-C	2.77 ± 0.05	4.1 ± 0.07	0.01371
	Nr of observation	7	2	

Three weeks before sample collection, both wild type and VDR-KO NMRI mice were fed with special foods containing high calcium. Serum cholesterol and HDL-C levels of both types of mice were determined. Data were analyzed by Mann-Whitney *U* test and presented as mean value ± standard error. The unit used in the table is mmol/L (SI Unit). To convert values from SI units to conventional units, divide by the conversion factor. For cholesterol and HDL-C, the convert factor is 0.0259.

Figure 1

ABCA1, ApoAI, LDLR, LXRβ and SREBP2 expression in the liver of wild type and VDR knockout mice. (A) Female and (B) Male. Data represented mean value ± standard error from quantitative real-time PCR results and statistically analyzed by Mann-Whitney *U* test. Mouse *β-actin* was used as housekeeping gene for the calculation of relative corresponding gene expression.

Figure 1A

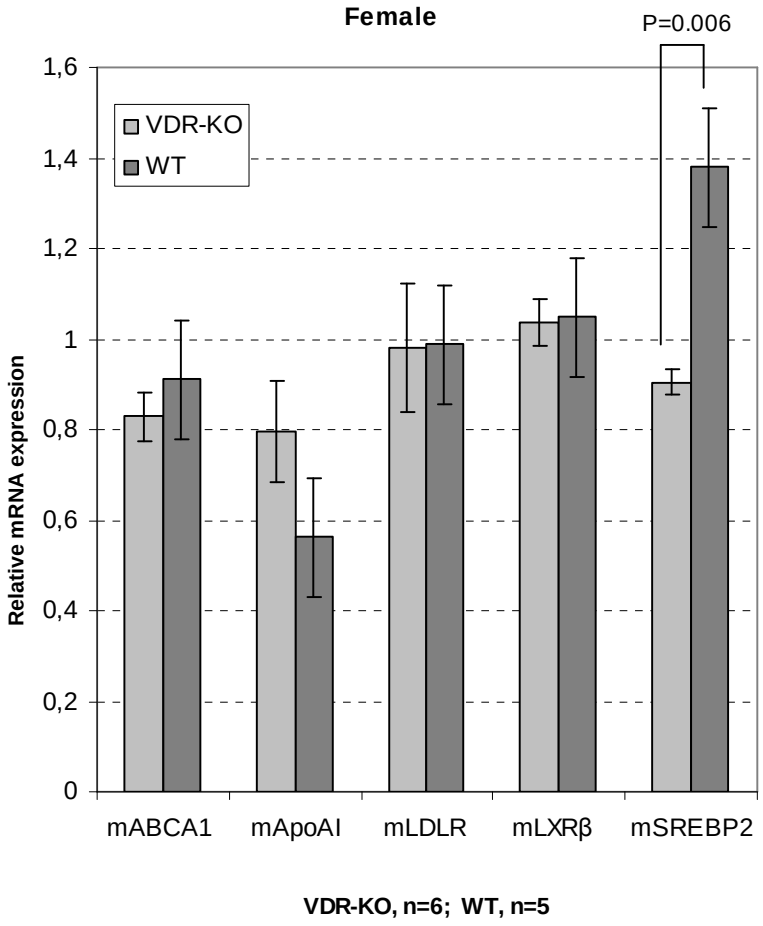
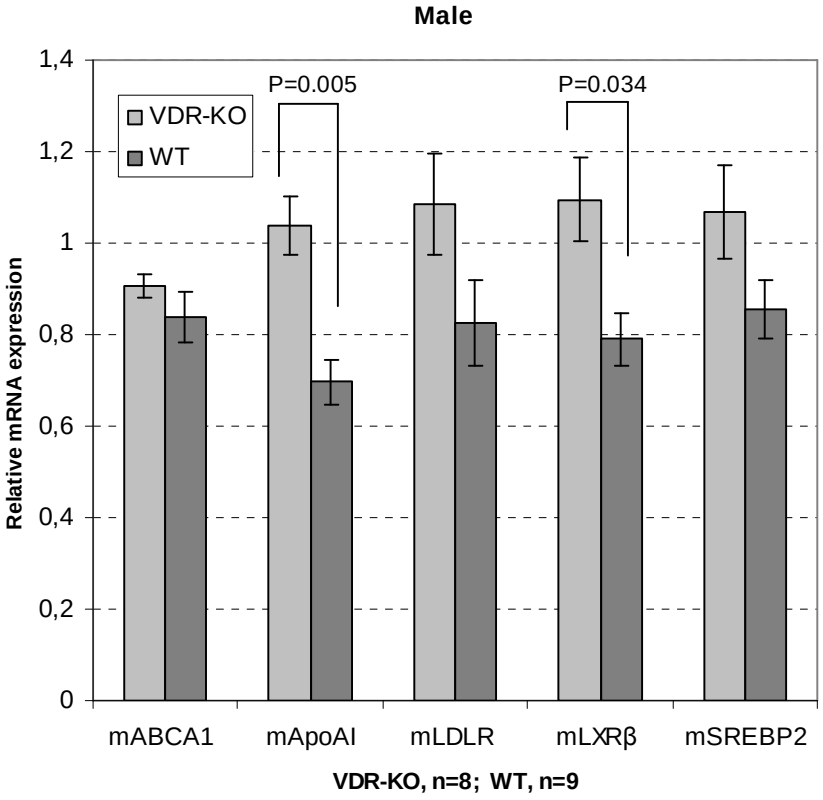


Figure 1B


Male
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Relative mRNA expression
VDR-KO
WT
P=0.005
P=0.034
mABCA1
mApoAI
mLDLR
mLXRβ
mSREBP2
VDR-KO, n=8; WT, n=9