YAN-RU LOU

Vitamin D_3 Metabolism in Normal and Malignant Human Prostate Cells

■

ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of Building B,
Medical School of the University of Tampere,
Medisiinarinkatu 3, Tampere, on December 16th, 2005, at 12 o'clock.

Acta Universitatis Tamperensis 1127

ACADEMIC DISSERTATION
University of Tampere, Medical School
Tampere University Hospital, Department of Clinical Chemistry
Finland

Supervised by
Professor Pentti Tuohimaa
University of Tampere

Reviewed by
Docent Anitta Mahonen
University of Kuopio
Professor Pirkko Vihko
University of Oulu

Distribution
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 3 3551 6055
Fax +358 3 3551 7685
taju@uta.fi
www.uta.fi/taju
http://granum.uta.fi

Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 1127
ISBN 951-44-6498-2
ISSN 1455-1616

Electronic dissertation
Acta Electronica Universitatis Tamperensis 498
ISBN 951-44-6499-0
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2005

CONTENTS

ABSTRACT

Vitamin D_3 is produced in the skin during exposure to sunlight and is then 25-hydroxylated in the liver, yielding the major circulating metabolite 25-hydroxyvitamin D_3 ($25OHD_3$). $25OHD_3$ is converted to 1α,25-dihydroxyvitamin D_3 [$1\alpha,25\text{-}(OH)_2D_3$] mainly in the kidney by $25OHD_3$-1α-hydroxylase (1α-hydroxylase). $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ are further hydroxylated into less active metabolites by $25OHD_3$-24-hydroxylase (24-hydroxylase, CYP24). Vitamin D_3 metabolites exert their effects by binding to the vitamin D receptor. 24-Hydroxylase is highly inducible by vitamin D_3 metabolites at transcriptional level and controls the biological action of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$. The active form of vitamin D_3, $1\alpha,25\text{-}(OH)_2D_3$ plays a central role in calcium homeostasis and at hypercalcemic concentrations it regulates the proliferation and differentiation of various cell types.

The present study was designed to investigate the role of vitamin D_3 metabolites and their enzymes in human primary prostate stromal and epithelial cells as well as in cancer cells. In addition, the crosstalk between vitamin D_3 and androgen as well as retinoic acid was investigated.

The present data show the expression of 1α-hydroxylase and 24-hydroxylase in the prostate. 1α-Hydroxylase is up-regulated by $25OHD_3$ in stromal cells. 24-Hydroxylase is up-regulated by $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ in epithelial and stromal cells. The transcriptional activity of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ in stromal cells is greatly increased in the presence of a 24-hydroxylase inhibitor, VID400.

The crosstalk between vitamin D_3 and 5α-dihydrotestosterone (DHT) or all-trans-retinoic acid (ATRA) was studied in prostate stromal and epithelial cells. DHT at a physiological concentration enhances the antiproliferative activities of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ by suppressing the expression of 24-hydroxylase in LNCaP cells. ATRA via retinoic acid receptor α (RARα) significantly decreases the expression of 24-hydroxylase mRNA induced by $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ in primary cultures of human prostate stromal cells P29SN and P32S but not in either primary culture of human prostate epithelial cells PrEC or cancer epithelial cells LNCaP and PC3. Cell proliferation study showed that the combined treatment of $1\alpha,25\text{-}(OH)_2D_3$ and a RARα-selective ligand, Am80 at 10 nM strongly inhibits cell proliferation whereas either alone has no effect.

By inhibiting 1α-hydroxylase enzyme activity, the induction of 24-hydroxylase mRNA by 250 nM $25OHD_3$ was clearly enhanced in stromal cells, suggesting that 1α-hydroxylation is not a prerequisite for the hormonal activity of $25OHD_3$. This finding that $25OHD_3$ at a physiological concentration possesses an inherent hormonal activity provides a novel view of the vitamin D_3 endocrine system and suggests that it could be used as an anticancer therapy. $1\alpha,25\text{-}(OH)_2D_3$ is inactive at its physiological concentrations but pharmacological concentrations are needed for induction of target gene expression and growth inhibition.

Altogether, the present study demonstrates a novel vitamin D_3 endocrine system mediated by $25OHD_3$ and provides feasible therapeutic approaches by using DHT or ATRA in combination

with vitamin D_3 metabolites at physiological concentrations. It also suggests that 24-hydroxylase is a key factor in inhibiting the action of vitamin D_3 metabolites in cancer chemoprevention and therapy.

ABBREVIATIONS

$1\alpha OHD_2$	1α-hydroxyvitamin D_2
$1\alpha OHD_3$	1α-hydroxyvitamin D_3
$1\alpha,25\text{-}(OH)_2D_3$	1α,25-dihydroxyvitamin D_3, calcitriol
$1\alpha,24,25\text{-}(OH)_3D_3$	1α,24,25-trihydroxyvitamin D_3
$24,25\text{-}(OH)_2D_3$	24,25-dihydroxyvitamin D_3
$25OHD_2$	25-hydroxyvitamin D_2
$25OHD_3$	25-hydroxyvitamin D_3, calcidiol
AF	activating function
ANOVA	analysis of variance
AR	androgen receptor
ATRA	all-trans-retinoic acid
BSA	bovine serum albumin
cAMP	cyclic 3',5'-adenosine monophosphate
CBP	cAMP response element-binding protein
C-terminus	carboxyl terminus
CYP24A1, 24-hydroxylase	$25OHD_3$-24-hydroxylase
CYP27A1	27-hydroxylase
CYP27B1, 1α-Hydroxylase	$25OHD_3$-1α-hydroxylase
DBD	DNA-binding domain
DBP	vitamin D binding protein
DCC-FBS	dextran-treated charcoal-stripped fetal bovine serum
DHT	5α-dihydrotestosterone
DRIP	VDR-interacting protein
ECL	enhanced chemiluminescence
ER	estrogen receptor
FBS	fetal bovine serum
HPLC	high-performance liquid chromatography
IDBP	intracellular vitamin D binding protein
IGF-1	insulin-like growth factor-1
IGFBP-3	insulin-like growth factor binding protein-3
Ig	immunoglobulin
K_d	dissociation constant
LBD	ligand-binding domain
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
NADPH	reduced nicotinamide-adenine dinucleotide phosphate
NCoR-1	nuclear receptor corepressor-1
N-terminal	amino terminal
OD	optical density
PBS	phosphate-buffered saline
PKA	protein kinase A
PKC	protein kinase C
PMCA	plasma membrane calcium ATPase
PPAR	peroxisome proliferator-activated receptor
PSA	prostate-specific antigen

PTH	parathyroid hormone
RAR	retinoic acid receptor
RPLP0	human acidic ribosomal phosphoprotein P0
rRNA	ribosomal RNA
RXR	retinoid X receptor
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SRC	steroid receptor coactivator
TBS	Tris-HCl buffered saline
TFIIB	transcription factor IIB
TGFβ	transforming growth factor β
TIF	transcriptional intermediary factor
TR	thyroid hormone receptor
TRPV	transient receptor potential cation channel
UV	ultraviolet
VDR	vitamin D_3 receptor
VDRE	vitamin D-response element
vs.	versus
YY1	Ying-Yang 1

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I Yan-Ru Lou, Ilkka Laaksi, Heimo Syvälä, Merja Bläuer, Teuvo L.J. Tammela, Timo Ylikomi and Pentti Tuohimaa (2004): 25-Hydroxyvitamin D_3 is an active hormone in human primary prostatic stromal cells.
I a: FASEB Journal 18(2):332-334 (Epub 4 Dec 2003)
I b: http://www.fasebj.org/cgi/doi/10.1096/fj.03-0140fje

II Yan-Ru Lou, Nadja Nazarova, Riikka Talonpoika, and Pentti Tuohimaa (2005): 5α-Dihydrotestosterone inhibits 1α,25-dihydroxyvitamin D_3-induced expression of CYP24 in human prostate cancer cells. Prostate 63(3):222-230 (Epub 10 Nov 2004).

III Yan-Ru Lou, Susanna Miettinen, Hiroyuki Kagechika, Hinrich Gronemeyer and Pentti Tuohimaa (2005): Retinoic acid via RARα inhibits the expression of 24-hydroxylase in human prostate stromal cells. Biochemical and Biophysical Research Communications 338(4):1973-1981 (Epub 7 Nov 2005).

IV Yan-Ru Lou and Pentti Tuohimaa (2006): Androgen enhances the antiproliferative activity of vitamin D_3 by suppressing 24-hydroxylase expression in LNCaP cells. Journal of Steroid Biochemistry & Molecular Biology 99(1) (In press).

INTRODUCTION

The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$], plays a crucial role in calcium homeostasis and regulates the proliferation and differentiation of various cell types. However, the clinical use of 1α,25-$(OH)_2D_3$ is limited because of the induction of hypercalcemia at a concentration to suppress cell proliferation. 1α,25-$(OH)_2D_3$ functions through its interaction with vitamin D_3 receptor (VDR) and heterodimer partner 9-cis retinoic acid receptor (RXR) to regulate target gene transcription.

1α,25-$(OH)_2D_3$ is generated from two sequential hydroxylations of vitamin D_3, which is either of nutritional origin or produced by its precursor, 7-dehydrocholesterol (provitamin D_3) in the skin upon exposure to sunlight, UVB (290-315 nm). 25-Hydroxylation is a prerequisite for the activation of vitamin D_3, yielding the prohormone 25-hydroxyvitamin D_3 ($25OHD_3$). $25OHD_3$ is the major circulating metabolite and a marker of the nutritional state. $25OHD_3$ is then 1α-hydroxylated by $25OHD_3$-1α-hydroxylase (1α-hydroxylase, CYP27B1), yielding 1α,25-$(OH)_2D_3$. The principle site of 1α-hydroxylase activity is the proximal tubules of the kidney. 1α-Hydroxylase has also been found in many extra-renal tissues and cells, such as skin, placenta, lung, colon, macrophages and prostate epithelial cells, which suggests an autocrine and/or a paracrine role of 1α,25-$(OH)_2D_3$. The catabolism of 1α,25-$(OH)_2D_3$ is initiated by a mitochondrial cytochrome P450 enzyme $25OHD_3$-24-hydroxylase (24-hydroxylase, CYP24A1). It hydroxylates $25OHD_3$ and 1α,25-$(OH)_2D_3$. 24-Hydroxylase is expressed predominantly in the kidney and also found in various vitamin D_3 target tissues. 24-Hydroxylase expression is up-regulated in all vitamin D_3 target cells by 1α,25-$(OH)_2D_3$ at transcriptional level through activation of two vitamin D-response elements (VDREs) in its promoter region. CYP24A1 is known to be the strongly induced gene, therefore, the induction of CYP24A1 expression is often used as an indicator of 1α,25-$(OH)_2D_3$ responsiveness. Amplification of the CYP24A1 gene region has been found in human breast cancer, ovarian cancer, and prostate cancer as well as mouse islet carcinoma. CYP24A1 is therefore considered a candidate oncogene because it can abrogate vitamin D_3-mediated growth control. Thus, the expression of 1α-hydroxylase and 24-hydroxylase is important in the metabolism of vitamin D_3, which in turn regulates the local concentrations of $25OHD_3$ and 1α,25-$(OH)_2D_3$. This thesis aimed to elucidate the role of $25OHD_3$, 1α,25-$(OH)_2D_3$, and their metabolizing enzymes in normal human prostate stromal and epithelial cells as well as in prostate cancer cells.

REVIEW OF THE LITERATURE

1. VITAMIN D

Vitamin D was discovered in the study of rickets. As early as 1822, a Polish physician, Sniadecki, observed and concluded that sunbathing cured rickets. Later it was demonstrated that exposure of the skin to UV radiation was responsible for the antirachitic activity (Huldschinsky 1919). After Mellanby's finding that cod liver oil containing vitamin A could cure the rachitic condition in dogs (1919), it was discovered that the antirachitic factor in cod liver oil was in fact not vitamin A, but a new vitamin designated vitamin D (McCollum et al. 1922). The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$], plays a key role in calcium homeostasis as well as in bone development and maintenance. Additionally, 1α,25-$(OH)_2D_3$ regulates the proliferation and differentiation of various cell types (Jones et al. 1998, Nagpal et al. 2005).

1.1. Metabolism of vitamin D

Vitamin D is not a true vitamin because its precursor can be produced upon the exposure of the skin to ultraviolet B (UVB). Vitamin D is a secosteroid (secosterol), which means that one of the rings in its cyclopentanoperhydrophenanthrene ring structure undergoes breakage of a carbon-carbon bond; for vitamin D, it is the 9,10 carbon-carbon bond of ring B (Figure 1). There are two forms of vitamin D with distinct structures. Vitamin D_2 (also known as ergocalciferol) is a natural form in plants, whereas vitamin D_3 (cholecalciferol) is synthesized by vertebrates (DeLuca 2004).

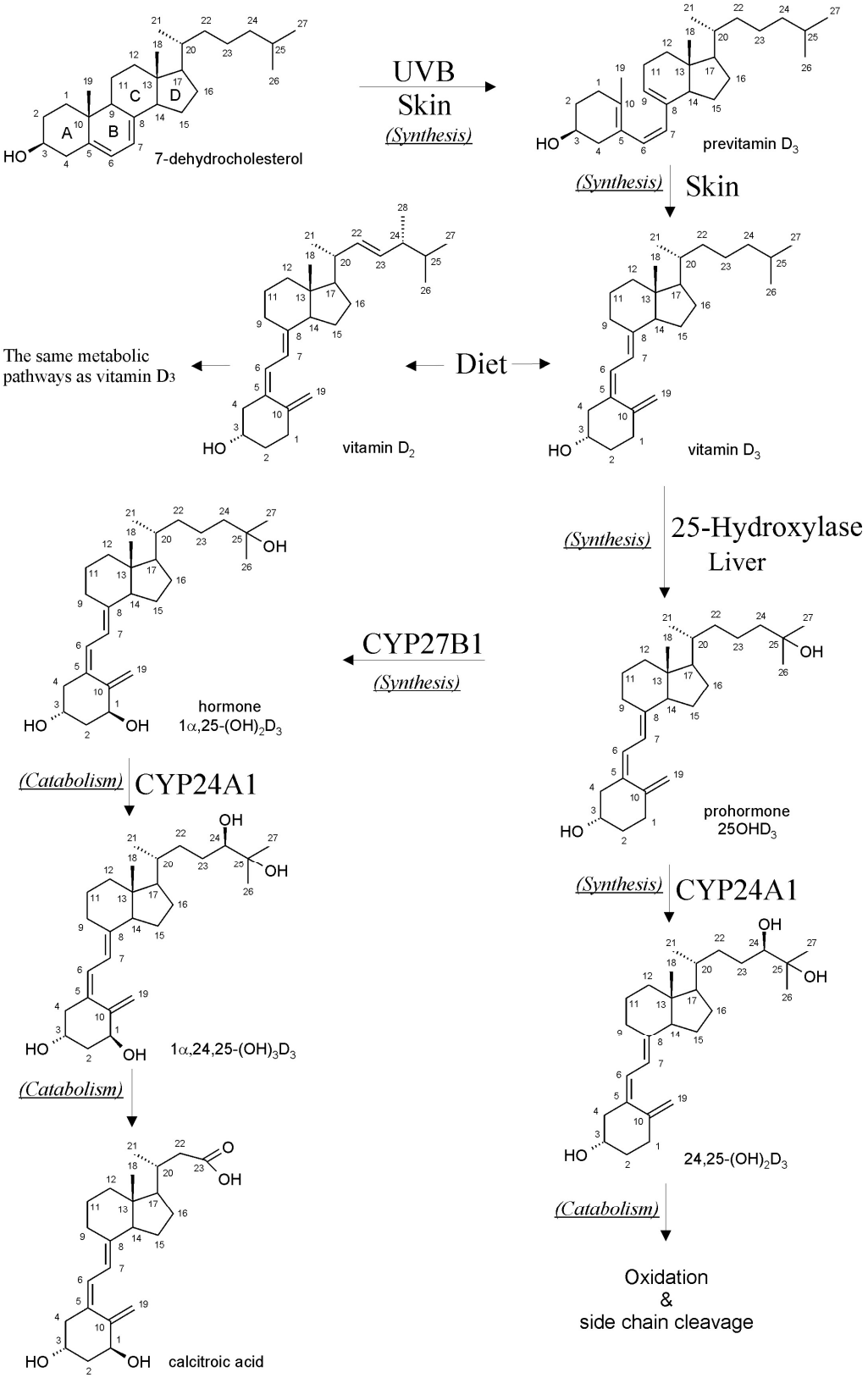


Figure 1. The metabolism of vitamin D_3.

1.1.1. Photosynthesis of vitamin D_3

In mammals vitamin D_3 is derived either from its cholesterol-like precursor in the skin or from nutritional sources. During the exposure of human skin to sunlight, UVB (290-315 nm) converts the precursor, 7-dehydrocholesterol (provitamin D_3) in the epidermis into previtamin D_3, which is then isomerized to vitamin D_3. Previtamin D_3 can also isomerize into biologically inactive photoisomers lumisterol and tachysterol in order to avoid vitamin D_3 intoxication (Bouillon et al. 1998). There are three factors affecting the output of vitamin D_3. The first factor is UVB, which is influenced by season, latitude, and air pollution (Webb et al. 1988, Agarwal et al. 2002). The second factor is the concentration of 7-dehydrocholesterol, which decreases with age (MacLaughlin and Holick 1985). The third factor is the skin. Sunscreen (Matsuoka et al. 1987), clothing (Matsuoka et al. 1992, Salih 2004), and increased melanin pigmentation (Clemens et al. 1982, Bell et al. 1985) can reduce the photosynthesis of vitamin D_3. Once vitamin D_3 is produced, it enters the extracellular fluid space, where it binds to vitamin D binding protein (DBP) in the circulation, and finally enters the bloodstream. Vitamin D_3 is then taken up either by the adipose tissue for storage or by the liver for further metabolism (Jones et al. 1998).

1.1.2. Vitamin D_3-25-hydroxylase

25-Hydroxylation is a prerequisite for the activation of vitamin D_3, yielding 25-hydroxyvitamin D_3 ($25OHD_3$). $25OHD_3$ is the major circulating metabolite and a marker of the nutritional state. The normal range of serum $25OHD_3$ concentrations is 25-137.5 nmol/L (Weaver and Fleet 2004), which varies with seasons among populations (Hine and Roberts 1994). However, it has been suggested that the adequate $25OHD_3$ concentrations would be 100-200 nmol/L (Zittermann 2003). The liver was shown to be the main site of 25-hydroxylation (Ponchon and DeLuca 1969, Ponchon et al. 1969). To date, three cytochrome P450 enzymes, CYP27A1, CYP2R1, and CYP3A4 have been reported to possess 25-hydroxylase activity. The first identified enzyme was a mitochondrial protein, sterol 27-hydroxylase (CYP27A1) (Cali and Russell 1991, Guo et al. 1993). It hydroxylates a variety of sterols at the C27 position. This process involves cytochrome P450 enzyme receiving two electrons sequentially from reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and a short transfer chain formed by ferredoxin reductase (adrenodoxin reductase) and ferredoxin (adrenodoxin). Mice with disrupted sterol 27-hydroxylase gene had normal plasma levels of cholesterol and $1\alpha,25\text{-}(OH)_2D_3$ (Rosen et al. 1998), indicating that sterol 27-hydroxylase is not critical for the maintenance of levels of vitamin D_3 metabolites in the circulation. Extra-hepatic tissues, such as kidney, skin, and intestine have been reported to possess CYP27A1 activity (Gascon-Barre et al. 2001, Schuessler et al. 2001, Theodoropoulos et al. 2003). CYP2R1 enzyme is a recently identified microsomal protein, vitamin D_3-25-hydroxylase (Cheng et al. 2003). Unlike sterol 27-hydroxylase, which shows specificity for vitamin D_3, CYP2R1 enzyme hydroxylates both vitamin D_2 and vitamin D_3. CYP27A1 hydroxylase is a low affinity, high capacity enzyme, whereas CYP2R1 hydroxylase is a high affinity, low capacity enzyme (Cheng et al. 2003). Therefore, the microsomal hydroxylase is capable of functioning at the physiological condition and is the major enzyme in 25-hydroxylation. CYP2R1 mRNA is abundant in the liver and testis, but the physiological significance of its expression in the testis remains unclear. A mutation in the CYP2R1 gene has been reported in a rickets patient (Cheng et al. 2004), emphasizing the important role of CYP2R1 enzyme in vitamin D metabolism. CYP3A4 is the liver microsomal enzyme with broad specificity. Although it was cloned in 1986, it has only recently been found

to 25-hydroxylate vitamin D_2, 1α-hydroxyvitamin D_2 (1αOHD_2), and 1α-hydroxyvitamin D_3 (1αOHD_3) in the liver (Gupta et al. 2004). Recombinant enzyme study shows that CYP3A4 is also a 24-hydroxylase for vitamin D_2, 1αOHD_2, and 1αOHD_3 (Gupta et al. 2005). Its activity has also been found in the intestine.

The regulation of 25-hydroxylation is not fully understood. CYP27A1 enzyme is down-regulated by 1α,25-$(OH)_2D_3$ in rat kidney and liver (Axen et al. 1995), and by 25OHD_3 and 1α,25-$(OH)_2D_3$ in rat intestine (Theodoropoulos et al. 2001). Growth hormone, insulin-like growth factor-1 (IGF-1), and the synthetic glucocorticoid, dexamethasone, increase the promoter activity of CYP27A1 (Araya et al. 2003). The microsomal enzymes CYP2R1 and CYP3A4 are so new findings that little are known about their regulation. CYP3A4 gene contains a vitamin D-response element (VDRE) and its expression is up-regulated by 1α,25-$(OH)_2D_3$ in small intestinal and colon cancer cells (Thompson et al. 2002). However, this vitamin D_3 receptor (VDR)-mediated regulation may not be meaningful in the liver, because of the undetectable level of VDR in the liver (Clemens et al. 1988).

1.1.3. 25-Hydroxyvitamin D_3-1α-hydroxylase

The next step is 25OHD_3-1α-hydroxylation catalyzed by 25OHD_3-1α-hydroxylase (1α-hydroxylase, CYP27B1), yielding the main active metabolite 1α,25-$(OH)_2D_3$. The normal physiological concentration of 1α,25-$(OH)_2D_3$ is 0.05-0.15 nmol/L (Mehta and Mehta 2002), which is 500-1000-fold lower than that of 25OHD_3. The serum 1α,25-$(OH)_2D_3$ concentration shows no seasonal variation (Hine and Roberts 1994). The principal site of 1α-hydroxylase activity is the proximal tubules of the kidney. 1α-Hydroxylase protein is a member of the cytochrome P450 superfamily localized to the inner mitochondrial membrane, where it hydroxylates 25OHD_3 at the C1α position. Enzymatic studies using a reconstituted system containing a membrane fraction of recombinant bacterial cells show that the 25-hydroxyl group is essential for 1α-hydroxylase activity and that the 24R-hydroxyl group enhances it, but the 23S-hydroxyl group reduces it (Sakaki et al. 1999b, Sawada et al. 1999). Human CYP27B1 was cloned by three separate groups (Fu et al. 1997, Monkawa et al. 1997, St-Arnaud et al. 1997). 1α-Hydroxylase-knockout mice exhibit a phenotype identical to that observed in human vitamin D-dependent rickets type I, a disease caused by mutations in CYP27B1 gene (Dardenne et al. 2001, Panda et al. 2001). Additionally, female infertility and abnormal immune function were observed in 1α-hydroxylase-ablated mice. Enriched calcium diet corrects the abnormal mineral ion homeostasis in 1α-hydroxylase-knockout mice, but it is less effective than 1α,25-$(OH)_2D_3$ in restoring bone growth (Dardenne et al. 2003a, Dardenne et al. 2003b), which reconfirms the important role of 1α-hydroxylase in calcium homeostasis. The renal 1α-hydroxylase is tightly controlled by several factors; the expression in the kidney is down-regulated by 1α,25-$(OH)_2D_3$, hypercalcemia, and hyperphosphatemia, and up-regulated by parathyroid hormone (PTH), calcitonin, hypocalcemia, and hypophosphatemia (Bland et al. 1999, Murayama et al. 1999, Zhang et al. 2002). A negative regulatory region to 1α,25-$(OH)_2D_3$ as well as a positive regulatory region to PTH and calcitonin have been identified in CYP27B1 gene promoter, indicating that the regulation of 1α-hydroxylase expression by 1α,25-$(OH)_2D_3$, PTH, and calcitonin takes place at transcriptional level (Brenza et al. 1998, Murayama et al. 1998, Kong et

al. 1999). In addition, the expression of 1α-hydroxylase decreases with age (Anderson et al. 2005).

The same 1α-hydroxylase has been found in many extra-renal tissues and cells (Zehnder et al. 2001), such as skin (Bikle et al. 1986), colon (Cross et al. 1997), intestine (Theodoropoulos et al. 2003), lung (Jones et al. 1999), placenta (Diaz et al. 2000b), macrophages (Monkawa et al. 2000), and prostate epithelial cells (Barreto et al. 2000), which supports the concept that 1α,25-$(OH)_2D_3$ acts as an autocrine and/or a paracrine factor. 1α-Hydroxylase gene amplification and mRNA splice variants have been found in malignant glioma (Maas et al. 2001). The regulation of the extra-renal 1α-hydroxylase is not identical to that in the kidney. Synthesis of 1α,25-$(OH)_2D_3$ is regulated by feedback mechanism in keratinocytes (Bikle et al. 1986), decidual cells (Delvin and Arabian 1987), lung cancer cells (Jones et al. 1999), macrophages (Monkawa et al. 2000), and prostate epithelial cells (Young et al. 2004), but it is unaffected by either phosphate, PTH, or calcium. Interestingly, the expression of CYP27B1 mRNA is unaffected by 1α,25-$(OH)_2D_3$ in lung cancer cells (Jones et al. 1999), macrophages (Monkawa et al. 2000) and keratinocytes (Xie et al. 2002). It has been elucidated that the decrease of 1α-hydroxylase activity is, in fact, due to the increased catabolism of both substrate $25OHD_3$ and product 1α,25-$(OH)_2D_3$ by 24-hydroxylase (Xie et al. 2002). Interferon γ also regulates the synthesis of 1α,25-$(OH)_2D_3$ in keratinocytes (Bikle et al. 1989) and macrophages (Kreutz et al. 1993, Dusso et al. 1997).

In contrast to the above-mentioned human vitamin D-dependent rickets type I, granulomatous diseases such as sarcoidosis are associated with a high serum concentration of 1α,25-$(OH)_2D_3$ caused by the elevated activity of 1α-hydroxylase in macrophages (Adams et al. 1983, Adams et al. 1985). A group of enzyme inhibitors has been developed. They include antifungal imidazole derivatives, such as ketoconazole and liarozole, both of which inhibit many P450 enzymes. Ketoconazole decreases the synthesis of 1α,25-$(OH)_2D_3$ and can be used in the treatment of sarcoidosis-associated hypercalcemia (Adams et al. 1990, Bia and Insogna 1991). A newly identified SDZ88-357, which specifically inhibits 1α-hydroxylase activity (Schuster et al. 2001b), might be a useful tool to study the metabolism of vitamin D_3.

1.1.4. 25-Hydroxyvitamin D_3-24-hydroxylase

The catabolism of 1α,25-$(OH)_2D_3$ is initiated by a mitochondrial cytochrome P450 enzyme $25OHD_3$-24-hydroxylase (24-hydroxylase, CYP24A1). It hydroxylates both $25OHD_3$ and 1α,25-$(OH)_2D_3$ at the C24 position, but neither vitamin D_3 nor $1\alpha OHD_3$ (Ohyama and Okuda 1991). Studies using recombinant bacterial and insect cells have shown that 24-hydroxylase is a multicatalytic enzyme catalyzing the reactions in the C-24/C-23 pathway to produce the final metabolites of $25OHD_3$ and 1α,25-$(OH)_2D_3$ (Akiyoshi-Shibata et al. 1994, Beckman et al. 1996, Sakaki et al. 1999a, Sakaki et al. 2000). 24-Hydroxylase displays 10-fold greater affinity for 1α,25-$(OH)_2D_3$ than $25OHD_3$ (Chen et al. 1993). DBP reduces the rate of $25OHD_3$ metabolism but not of 1α,25-$(OH)_2D_3$ metabolism (Masuda et al. 2004). 24-Hydroxylase is expressed predominantly in the kidney and also found in various vitamin D_3 target tissues. However, it is absent from the liver (Armbrecht and Boltz 1991). Recently, a CYP24A1 splice variant, designated hCYP24-SV, has been characterized in the kidney, placenta, keratinocytes, and

macrophages (Ren et al. 2005). hCYP24-SV lacks the mitochondrial targeting sequence and the variant protein is therefore functionally inactive (Ren et al. 2005). This truncated 24-hydroxylase may contribute to elevated levels of 1α,25-$(OH)_2D_3$ (Ren et al. 2005). The increase in renal CYP24A1 mRNA and activity with age has been reported in rats, suggesting that the increased renal catabolism of 1α,25-$(OH)_2D_3$ may contribute to the decrease in serum level of 1α,25-$(OH)_2D_3$ (Johnson et al. 1995, Matkovits and Christakos 1995a, Anderson et al. 2005). 24-Hydroxylase expression is up-regulated in all vitamin D_3 target cells by 1α,25-$(OH)_2D_3$ at transcriptional level through activation of two VDREs in its promoter region (Chen and DeLuca 1995). CYP24A1 is the most strongly regulated gene by 1α,25-$(OH)_2D_3$, therefore the induction of CYP24A1 expression is often used as an indicator of 1α,25-$(OH)_2D_3$ responsiveness. The major role of 24-hydroxylase appears to be the inactivation of 1α,25-$(OH)_2D_3$ to maintain systemic calcium homeostasis and to turn off the local effects of 1α,25-$(OH)_2D_3$. Studies have shown that the induction of 24-hydroxylase by 1α,25-$(OH)_2D_3$ requires new protein synthesis (Armbrecht et al. 1997, Zierold et al. 2002, Zierold et al. 2003). To date, no genetic disorder of 24-hydroxylase in humans has been reported. However, amplification of the CYP24A1 gene region has been found in human breast cancer (Kallioniemi et al. 1994, Tanner et al. 1995), ovarian cancer (Tanner et al. 2000), and prostate cancer (Wolter et al. 2002) and mouse islet carcinoma (Hodgson et al. 2001). CYP24A1 is, therefore, considered a candidate oncogene (Albertson et al. 2000) because it can abrogate vitamin D_3-mediated growth control. Studies on 24-hydroxylse-knockout mice and VDR-knockout mice have led to a better understanding of the importance of 24-hydroxylase and VDR in 1α,25-$(OH)_2D_3$ catabolism. 24-Hydroxylase-knockout mice are incapable of excreting exogenous 1α,25-$(OH)_2D_3$, however, the surviving knockout mice have surprisingly low basal levels of 1α,25-$(OH)_2D_3$ compared to the wild-type controls (St-Arnaud et al. 2000). Further studies (Masuda et al. 2005) demonstrated that there was no alternative pathway of 1α,25-$(OH)_2D_3$ catabolism and the surviving knockout mice may adapt, in part, by suppressing 1α,25-$(OH)_2D_3$ synthesis because 1α-hydroxylase mRNA in the kidney was undetectable. No C23/C26 oxidation products were detected in keratinocytes from Cyp24a1-knockout mice, emphasizing the importance of the multiple roles of CYP24A1. In addition, keratinocytes from VDR-knockout mice showed a complete block of 1α,25-$(OH)_2D_3$ catabolism. It can be concluded that both 1α-hydroxylase and 24-hydroxylase regulate 1α,25-$(OH)_2D_3$ homeostasis through VDR.

Other factors such as PTH, calcium, and calcitonin can also regulate CYP24A1. PTH suppresses 24-hydroxylase mRNA by altering its stability in the kidney cells (Zierold et al. 2001). However PTH enhances the 1α,25-$(OH)_2D_3$-dependent induction of 24-hydroxylase in osteoblastic cells (Krishnan et al. 1995, Armbrecht et al. 1998) and its action is potentiated by insulin (Armbrecht et al. 1996). But other group has reported no PTH effect on 1α,25-$(OH)_2D_3$-dependent induction of 24-hydroxylase in bone cells (Nishimura et al. 1994). PTH has no effect on 24-hydroxylase in the intestine (Shinki et al. 1992) because of the absence of PTH receptors. Low calcium indirectly suppresses renal 24-hydroxylase activity due to secondary hyperparathyroidism but not bone 24-hydroxylase activity (Nishimura et al. 1994). Calcium exhibits no effect on the expression of intestinal 24-hydroxylase (Lemay et al. 1995). Calcitonin causes suppression of 24-hydroxylase mRNA expression and activity in the intestine (Beckman et al. 1994). The effect of calcitonin in the kidney has recently been described. Unlike in the intestine, calcitonin

stimulates renal 24-hydroxylase-promoter expression and such action is suggested to be very important in hypercalcemic state (Gao et al. 2004). Interferon γ abrogates 1α,25-$(OH)_2D_3$-mediated induction of 24-hydroxylase in human monocytes and macrophages, which is one of the reasons for the hypercalcemia of various granulomatoses (Dusso et al. 1997).

One of the interesting fields in the study of vitamin D_3 is the development of 24-hydroxylase inhibitors, which are not only useful tools to study 1α,25-$(OH)_2D_3$ catabolism but also to enhance 1α,25-$(OH)_2D_3$ action. The high basal and self-induced expression of 24-hydroxylase may cause 1α,25-$(OH)_2D_3$-resistance. Ketoconazole increases 1α,25-$(OH)_2D_3$-dependent induction of VDR via decreasing 1α,25-$(OH)_2D_3$ catabolism (Reinhardt and Horst 1989). Liarozole acts synergistically with 1α,25-$(OH)_2D_3$ in 1α,25-$(OH)_2D_3$-resistant cells (Ly et al. 1999). VID400, a selective inhibitor of 24-hydroxylase is a valuable tool to explore distinct functions of different metabolites (Schuster et al. 2001a, Schuster et al. 2001b).

Because the serum concentration of $25OHD_3$ is 500-1000-fold higher than that of 1α,25-$(OH)_2D_3$, the major 24-hydroxyl metabolite in blood is 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] despite the 10-fold greater affinity of 24-hydroxylase to 1α,25-$(OH)_2D_3$. The physiological functions of 24,25-$(OH)_2D_3$ are not clear and need to be defined. 24,25-$(OH)_2D_3$ was first shown to be essential for bone formation in 1978 (Ornoy et al. 1978). Later, several *in vivo* studies have shown that massive doses of 24,25-$(OH)_2D_3$ increase bone volume and strength in rats, rabbits, and mice (Nakamura et al. 1987, Nakamura et al. 1988, Nakamura et al. 1989, Nakamura et al. 1992, Ono et al. 1996), indicating its role in bone formation at pharmacological concentrations. However, another group showed that 1α,25-$(OH)_2D_3$, but not 24,25-$(OH)_2D_3$, is necessary and sufficient for normal bone growth and development in rats (Parfitt et al. 1984). Other physiological functions of 24,25-$(OH)_2D_3$ are associated with fracture healing (Seo et al. 1997, Seo and Norman 1997), embryo development (Henry and Norman 1978, Norman et al. 1983), and cartilage development (Schwartz et al. 1995b). A membrane receptor for 24,25-$(OH)_2D_3$ has been proposed (Pedrozo et al. 1999). By using 24-hydroxylase-knockout mice, the role of 24,25-$(OH)_2D_3$ has been clarified. The lack of 24-hydroxylase activity during development results in impaired intramembranous bone mineralization. This phenotype was not rescued by treatment with 24,25-$(OH)_2D_3$, but rather by crossing the 24-hydroxylse-mutant mice with VDR-knockout mice, demonstrating that deficient mineralization of intramembranous bone in 24-hydroxylase-ablated mice is due to elevated 1α,25-$(OH)_2D_3$ acting via VDR, not to the absence of 24,25-$(OH)_2D_3$ (St-Arnaud et al. 2000). The authors concluded that 24-hydroxylase plays an important role in regulating 1α,25-$(OH)_2D_3$ homeostasis and 24,25-$(OH)_2D_3$ is a dispensable metabolite during bone development. The interaction between 1α,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ is also of interest. 24,25-$(OH)_2D_3$ at pharmacological concentrations suppresses 1α,25-$(OH)_2D_3$-stimulated osteoclast formation (Yamato et al. 1993) and the rapid action of 1α,25-$(OH)_2D_3$ on calcium transport (Takeuchi and Guggino 1996, Nemere 1999). The regulation of vitamin D_3 metabolic pathways is illustrated in Figure 2.

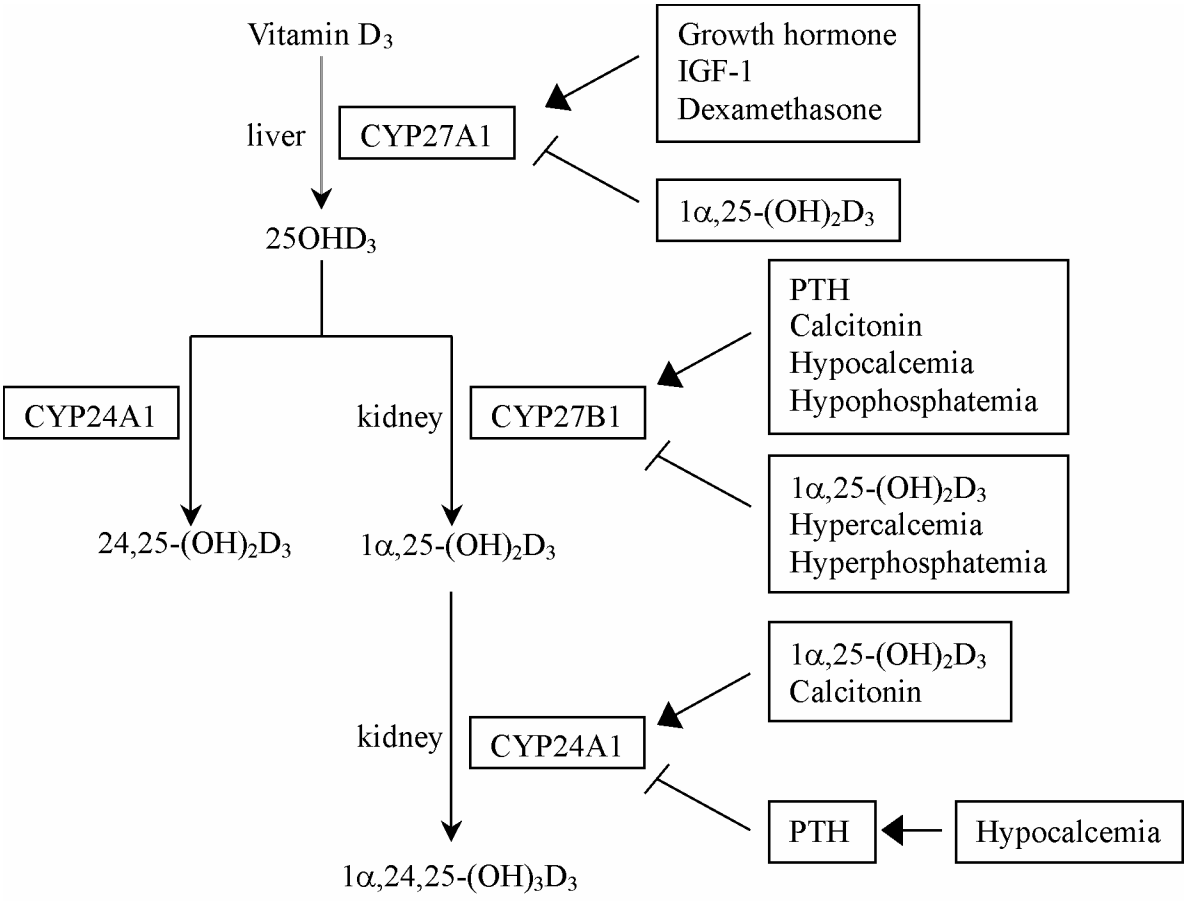


Figure 2. The regulation of vitamin D_3 metabolic pathways

1.2. Transport of vitamin D_3 metabolites

The bioavailability of vitamin D_3 metabolites is dependent on their rates of metabolism and catabolism, transport, cellular uptake, and intracellular trafficking. A serum protein called vitamin D binding protein (DBP, initially named group-specific component) plays a central role in the transportation of vitamin D metabolites and consequently regulates their storage, half-lives, and cellular uptake. DBP is predominantly expressed and secreted by the liver. DBP binds to 88% of serum $25OHD_3$ with a high affinity (5×10^{-8} M) and to 85% of serum $1\alpha,25\text{-}(OH)_2D_3$ with a lower affinity (4×10^{-7} M) (White and Cooke 2000). DBP has a ligand-binding domain that is distinct from that of VDR (Mizwicki and Norman 2003). It is believed that vitamin D_3 metabolites with the higher affinity for DBP possess longer half-lives, slower clearance rate, and poorer access to target cells (Jones et al. 1998). Therefore, the binding affinity for DBP is one of the factors to be considered in the design of vitamin D_3 analogs. DBP also functions in receptor-mediated endocytosis, in which $25OHD_3$-DBP complex is taken up in the proximal renal tubular cells by endocytic receptor megalin (Nykjaer et al. 1999). This process affects vitamin D renal metabolism and consequently, calcium homeostasis (Leheste et al. 2003). Once inside the target cells, how the vitamin D_3 metabolites reach mitochondria to bind their hydroxylases or nuclei to bind their receptors is not fully understood. Microtubule-based transport has been found to mediate the synthesis and nuclear activity of $1\alpha,25\text{-}(OH)_2D_3$ by regulating the intracellular trafficking of $25OHD_3$ (Kamimura et al. 1995). A group of intracellular vitamin D binding

proteins (IDBPs) in New World primates has been identified (Gacad et al. 1997). IDBPs are highly homologous to human 70-kDa heat shock proteins and bind $25OHD_3$ better than $1\alpha,25\text{-}(OH)_2D_3$ (Gacad and Adams 1998). It has been found that IDBPs increase VDR-mediated transactivation (Wu et al. 2000) and $1\alpha,25\text{-}(OH)_2D_3$ synthesis (Wu et al. 2002) by controlling intracellular localization of vitamin D metabolites.

1.3. Mechanisms of vitamin D_3 actions

1.3.1. Nuclear receptor and genomic actions

1.3.1.1. VDR

Most of the biological activities of vitamin D_3 metabolites are mediated by a nuclear protein, VDR, which was discovered over 30 years ago (Haussler et al. 1968). VDR was first cloned from chicken (McDonnell et al. 1987), followed by cloning from human, rat, and mouse (Baker et al. 1988, Burmester et al. 1988, Kamei et al. 1995). VDR is a ligand-activated transcription factor belonging to the nuclear receptor superfamily that includes receptors for estrogen, androgen, glucocorticoids, progesterone, thyroid hormone, and retinoids (Evans 1988). VDR exhibits the greatest similarity to the retinoic acid receptor (RAR) and thyroid hormone receptor (TR) (Jones et al. 1998). Like the other nuclear receptors, VDR has an A/B domain, a DNA-binding domain (DBD, C-domain), a hinge domain (D-domain), and a ligand-binding domain (LBD, E-domain) (DeLuca 2004, Lin and White 2004). The major steps of VDR action in regulating gene transcription include ligand binding to LBD, heterodimerization with the retinoid X receptor (RXR), DNA binding to the heterodimer VDR-RXR, and cofactor binding to LBD.

Unlike other nuclear receptors [estrogen receptor (ER), RXR and RAR, etc.], which contain a ligand-independent activating function-1 (AF-1) domain in their A/B domain, VDR lacks AF-1 domain in its A/B domain (Issa et al. 1998). DBD is the most conserved domain throughout the nuclear receptor family. The DBD of VDR recognizes specific DNA sequences through two zinc finger motifs that form two α-helices. The specific DNA sequences called VDREs are usually found in the promoter regions of target genes. It is known that the 5'-half-site of the VDRE binds RXR and the 3'-half-site of the VDRE binds VDR (DeLuca 2004). Some of the vitamin D-regulated genes are summarized in Table 1. As mentioned in Section 1.1.4, 24-hydroxylase is the most powerfully up-regulated gene.

Table 1. Vitamin D regulated genes (* containing VDRE; # containing a synthetic vitamin D analog responsive element; § no identified VDRE)

Category	Up-regulated	Down-regulated
Mineral and bone-related genes	Osteocalcin * (Kerner et al. 1989, Morrison et al. 1989)	Type I collagen * (Pavlin et al. 1994)
	Osteopontin * (Noda et al. 1990)	PTH * (Demay et al. 1992)
	Calbindin D_{9k} * (Darwish and DeLuca 1992)	PTH-related peptide * (Falzon 1996)
	Calbindin D_{28k} * (Gill and Christakos 1993)	Bone sialoprotein * (Li and Sodek 1993)
	RANKL * (Kitazawa and Kitazawa 2002, Kitazawa et al. 2003)	
	Carbonic anhydrase II * (Quelo et al. 1998)	
	Type II Na^+-dependent Pi transporter * (Taketani et al. 1998)	
	Na^+-sulfate cotransporter Nas1 * (Dawson and Markovich 2002)	
	TRPV5 § (Hoenderop et al. 2001)	
	TRPV6 § (Nijenhuis et al. 2003)	
Metabolism-related genes	CYP24A1 * (Chen and DeLuca 1995)	CYP27B1 * (Murayama et al. 1998)
	CYP3A4 * (Makishima et al. 2002)	CYP27A1 § (Axen et al. 1995)
	CYP3A1 * (Makishima et al. 2002)	
	CYP3A11 * (Makishima et al. 2002)	
	17β-hydroxysteroid dehydrogenase (hsd17b2) * (Wang et al. 2005)	
Cytokine and growth factor-related genes	IGFBP-3 * (Peng et al. 2004)	RelB * (Dong et al. 2003)
	PDGF-A * (Pedigo et al. 2003)	EGFR * (McGaffin et al. 2004)
	Insulin receptor * (Maestro et al. 2003)	IL-2 § (Manolagas et al. 1985)
	TGF-β2 * (Wu et al. 1999)	IFN-γ § (Cippitelli and Santoni 1998)
	TNFα * (Hakim and Bar-Shavit 2003)	IL-12 § (D'Ambrosio et al. 1998)

	IGFBP-5 § (Schmid et al. 1996)	
	TNF receptor 1 § (Mathiasen et al. 2001)	
	TGF-β1 § (Koli and Keski-Oja 1995)	
Cell cycle-related genes	$p21^{waf1}$ * (Liu et al. 1996a)	c-myc # (Okano et al. 1999)
	Cyclin C * (Sinkkonen et al. 2005)	
	$p27^{kip1}$ § (Wang et al. 1996)	
Apoptosis-related genes	Cathepsin B § (Simboli-Campbell et al. 1996)	Bcl-2 § (James et al. 1998)
	Clusterin § (Simboli-Campbell et al. 1996)	
	Bak § (Diaz et al. 2000a)	
Differentiation-related genes	Involucrin * (Bikle et al. 2002)	
	Phospholipase C-γ1 * (Xie and Bikle 1997)	
	Calcium sensing receptor § (Chakrabarty et al. 2005)	
Cell adhesion-related genes	β3 integrin * (Cao et al. 1993)	
	Fibronectin * (Polly et al. 1996)	
	E-cadherin § (Campbell et al. 1997)	
Other genes	Multidrug resistance-associated protein-3 * (McCarthy et al. 2005)	
	PPARδ * (Dunlop et al. 2005)	

The DBD is also responsible for the nuclear localization (Hsieh et al. 1998). The hinge domain of VDR confers flexibility to the protein in structural conformation upon ligand activation. Deletion of a few amino acids from the hinge region reduces the transcriptional activation of VDR *in vivo* (Shaffer et al. 2005). The LBD is a multifunctional domain and varies between nuclear receptors. The functions of the LBD in VDR involve binding of ligands, heterodimerization with RXR and binding of cofactors. $1\alpha,25\text{-}(OH)_2D_3$ has the greatest binding affinity to VDR (K_d = 90-300 pM for rat VDR) (Walters 1992). $25OHD_3$ binds to human VDR approximately 50 times less (Bouillon et al. 1995) and to chicken VDR 150-667 times less effectively (Brumbaugh and Haussler 1974, Bouillon et al. 1995). The study of the ligand binding pocket has led to development of synthetic $1\alpha,25\text{-}(OH)_2D_3$ analogues. Ligand binding to VDR results in heterodimerization with RXR. The dimerization interfaces have been found in the DBD and LBD domains (Issa et al. 1998, Brown et al. 1999). The DBD of VDR does not dimerize with the DBD of RXR in the absence of VDRE (Rastinejad 2001), but the dimerization of LBDs, in some cases, is DNA-independent (Khorasanizadeh and Rastinejad 2001). Ligand binding to VDR also causes the conformational change at the C-terminus of the protein, a ligand-dependent activating function-2 (AF-2) domain, which then recruits transcription factors, termed

coregulators. Nuclear receptor coregulators are coactivators and corepressors. Coregulators contribute to histone modifications, chromatin remodeling, recruitment of RNA polymerase and accessory factors, and receptor localization (McKenna et al. 1999, McKenna and O'Malley 2002). The known coactivators involved in VDR action are such as the transcription factor IIB (TFIIB), the VDR-interacting protein (DRIP), the vitamin D coactivator NCoA-62, steroid receptor coactivator (SRC-1), transcriptional intermediary factor 1 (TIF1), and the cyclic AMP response element-binding protein CBP/p300 (Rachez and Freedman 2000). The corepressors known to be involved in VDR action are nuclear receptor corepressor-1 (NCoR-1) (Tagami et al. 1998), NCoR-2 (Tagami et al. 1998), the silencing mediator of retinoic acid and thyroid hormone receptor (Li et al. 1997a), Alien (Polly et al. 2000), and hairless (Hsieh et al. 2003).

Activation of nuclear receptors normally requires ligand binding. It has been shown that some nuclear receptors can act ligand-independently. In the absence of ligands, phosphorylation may activate VDR-mediated transcription (Matkovits and Christakos 1995b). In the presence of the transcription factor Ets-1, VDR ligand-independently stimulates the prolactin promoter (Tolon et al. 2000). Alopecia has been observed in VDR knockout mice (Li et al. 1997b, Yoshizawa et al. 1997) and some kindreds with vitamin D-dependent rickets type II (Tsuchiya et al. 1980, Chen et al. 1984), but not in 1α-hydroxylase knockout mice (Dardenne et al. 2001, Panda et al. 2001), kindreds with vitamin D-dependent rickets type I, nor in patients with dietary vitamin D deficiency. Normalization of mineral homeostasis cannot prevent alopecia (Li et al. 1998). All these data strongly suggest that normal hair growth requires VDR, regardless of vitamin D_3 status. Recent investigation revealed that the maintenance of hair follicle homeostasis by VDR does not require ligand-dependent transactivation, but rather is mediated by VDR in a ligand-independent way (Chen et al. 2001, Skorija et al. 2005).

1.3.1.2. Regulation of nuclear action of VDR

The nuclear action of VDR may be influenced by availability of ligands, VDR, RXR, coregulators, and VDRE.

Firstly, the availability of ligands is affected by their metabolism, catabolism, and bioavailability.

Secondly, the status of VDR may vary due to the regulation of its expression, phosphorylation, nuclear localization, occupancy with other proteins and possibly polymorphisms. (I) VDR is widely expressed in various tissues. N-terminal variants of human VDR proteins, generated by tissue-specific promoters, have been described (Crofts et al. 1998, Sunn et al. 2001). These variants could contribute to the differential responsiveness to ligands in various tissues and cells. The expression of VDR can be regulated by its cognate ligands (Christakos et al. 1996), retinoic acid (Chen and Feldman 1985), activation of protein kinase A (PKA) (Krishnan and Feldman 1992) and protein kinase C (PKC) (Krishnan and Feldman 1991), PTH (Krishnan et al. 1995), DHT (Ahonen et al. 2000b), estrogens (Liel et al. 1992), and glucocorticoid (Chen et al. 1983). The analysis of human VDR gene shows a retinoic acid response site in the exon 1C and no VDRE (Miyamoto et al. 1997). Homologous up-regulation of VDR is tissue specific (Gensure et al. 1998) and, instead of appearing at the transcriptional level (Wiese et al. 1992); it is, rather, the result of blocking ubiquitin/proteasome-mediated degradation (Li et al. 1999a). (II) PKA, PKC, and casein kinase II are able to phosphorylate VDR (Brown et al. 1999). It has been shown that the ligand binding increases phosphorylation of VDR (Brown and DeLuca 1990).

Phosphorylation of VDR can either enhance (Barletta et al. 2002) or suppress (Hsieh et al. 2004) the VDR-mediated transcription. (III) RXR promotes nuclear accumulation of unliganded VDR, which correlates with an increase in basal transcriptional activity (Prufer et al. 2000). (IV) It has recently been discovered that direct protein-protein interactions between activated Stat1 and the DBD of VDR impair VDR-RXR binding to human 24-hydroxylase VDRE (Vidal et al. 2002). This mechanism delineates how interferon γ antagonizes the 1α,25-$(OH)_2D_3$-VDR transcriptional activation of 24-hydroxylase (Dusso et al. 1997). (V) The VDR polymorphisms have been suggested to be associated with bone-related features (Morrison et al. 1992, Morrison et al. 1994, Suarez et al. 1997, Carling et al. 1998, Tao et al. 1998, Uitterlinden et al. 2001). However, other studies do not support this relationship (Hustmyer et al. 1994, Lim et al. 1995, Garnero et al. 1996, Houston et al. 1996, Ensrud et al. 1999).

Thirdly, the status of RXR may vary due to its cognate ligand binding, its phosphorylation and occupancy with other receptor partners (RXR, RAR, TR, or PPAR, etc.). The liganded RXR may decrease (MacDonald et al. 1993) or increase (Giguere 1994) the action of VDR. Phosphorylation of RXRα can decrease 1α,25-$(OH)_2D_3$-dependent activity (Solomon et al. 1999). Transcriptional interference between actions of VDR and other nuclear receptors may occur due to competition for RXR (Giguere 1994, Raval-Pandya et al. 1998).

Fourthly, coregulators may be affected by their tissue specificity, occupancy with other nuclear receptors or regulators and perhaps by signal transduction pathways. TFIIB cell-type specifically regulates 1α,25-$(OH)_2D_3$-dependent transcription (Blanco et al. 1995). Competition between VDR and other nuclear receptors for the common coactivators could reduce the transcriptional activity of VDR as seen in the transcriptional interference between progesterone receptor and estrogen receptor (Meyer et al. 1989). In addition, a ubiquitous regulator Ying-Yang 1 (YY1) may also sequester TFIIB/CBP (Usheva and Shenk 1994, Guo et al. 1997, Raval-Pandya et al. 2001). SRC-1 is phosphorylated through mitogen-activated protein kinase (MAPK), which in turn regulates progesterone receptor-mediated activation (Rowan et al. 2000a, Rowan et al. 2000b). Activation of MAPK pathway may also influence VDR-mediated activation (Barletta et al. 2004). Phosphorylation has been reported to enhance VDR-mediated transcription by increasing interaction between VDR and DRIP205 (Barletta et al. 2002).

Fifthly, VDR action may be suppressed through competition for VDRE with a ubiquitous regulator YY1 (Guo et al. 1997) or by the VDRE-binding protein in New World primates (Chen et al. 2000a) and heterogeneous nuclear ribonucleoprotein in humans (Chen et al. 2003b).

1.3.2. Membrane receptor and non-genomic actions

In addition to the 1α,25-$(OH)_2D_3$-mediated genomic actions of nuclear VDR, 1α,25-$(OH)_2D_3$ also produces rapid biological responses involving activation of protein kinases and regulation of ion channels (Norman et al. 2002, Fleet 2004). There is some evidence supporting this concept. First, a putative membrane VDR has been found in basal-lateral membranes of chick intestinal epithelium (Nemere et al. 1994), rat costochondral resting zone and growth zone cartilage cells (Nemere et al. 1998) and human tooth and bone (Mesbah et al. 2002). The 1α,25-$(OH)_2D_3$ membrane-associated, rapid-response sterol-binding (1α,25-$(OH)_2D_3$-MARRS) protein has been found to be involved in phosphate transport in the chick duodenum and it is identical to the multifunctional protein endoplasmic reticulum protein ERp57 (Nemere et al. 2004). In addition,

non-genomic action of 1α,25-$(OH)_2D_3$ was found in nuclear VDR-knockout mice (Boyan et al. 2003, Wali et al. 2003). Some vitamin D analogs produce non-genomic action without having genomic effects because of the poor ability to bind to nuclear VDR (Norman et al. 1993, Dormanen et al. 1994). Second, the classical VDR has been shown to be associated with caveolae-enriched plasma membranes (Huhtakangas et al. 2004). Studies have shown that nuclear VDR had a role in non-genomic action (Nguyen et al. 2004, Zanello and Norman 2004).

2. BIOLOGICAL ACTIONS OF VITAMIN D_3

2.1. 1α,25-Dihydroxyvitamin D_3-mediated endocrine system

1α,25-$(OH)_2D_3$ plays a major role in modulating calcium homeostasis, bone development, and mineralization. There are three main actions of 1α,25-$(OH)_2D_3$ involved in calcium homeostasis. First, interaction of 1α,25-$(OH)_2D_3$ with VDR increases intestinal calcium absorption by stimulating three types of proteins (Van Cromphaut et al. 2001): (1) two calcium channel proteins, transient receptor potential cation channel 5 (TRPV5, former name epithelial calcium channel 1) (Muller et al. 2000), and TRPV6 (former names calcium channel CaT1 and epithelial calcium channel 2) (Peng et al. 2000); (2) an intracellular calcium transfer protein, calcium binding protein (calbindin D_{9k}) (Howard et al. 1992, Jeung et al. 1992); (3) a calcium extrusion protein, plasma membrane calcium ATPase 1 isoform 1b ($PMCA_{1b}$) (Verma et al. 1988, Kutuzova and Deluca 2004). Second, 1α,25-$(OH)_2D_3$ increases calcium reabsorption in the distal renal tubule by stimulating TRPV5 (Hoenderop et al. 2001), TRPV6 (Nijenhuis et al. 2003), and calbindin D_{28K} (Van Baal et al. 1996). Third, in the absence of intestinal calcium absorption, 1α,25-$(OH)_2D_3$ maintains serum calcium level by stimulating osteoblasts to produce receptor activator of nuclear factor-κB ligand (RANKL, other names tumor necrosis factor ligand 11, osteoprotegerin ligand, and osteoclast differentiation factor) (Palmqvist et al. 2002, Bergh et al. 2004), leading to osteoclastogenesis and bone resorption (Hofbauer and Heufelder 2001). The latter two actions are accompanied by PTH action. Calcium-sensing receptor in the parathyroid gland senses serum calcium concentration. When serum calcium concentration is low, the calcium-sensing receptor stimulates PTH secretion, which further, increases calcium reabsorption and 1α-hydroxylase activity in the kidney as well as induces calcium release from the bone matrix (DeLuca 2004, Hoenderop et al. 2005). 1α,25-$(OH)_2D_3$ suppresses the synthesis of PTH and parathyroid cell growth (Cantley et al. 1985, Szabo et al. 1989). Both VDR- and 1α-hydroxylase-knockout mice develop hypocalcemia after weaning (Li et al. 1997b, Yoshizawa et al. 1997, Dardenne et al. 2001, Panda et al. 2001), emphasizing the important role of the 1α,25-$(OH)_2D_3$/VDR system in calcium homeostasis.

The action of 1α,25-$(OH)_2D_3$ in bone growth and mineralization is related to intestinal absorption of calcium and regulation of bone cell differentiation and function. Both VDR- and 1α-hydroxylase-knockout mice exhibit severely impaired bone formation (Li et al. 1997b, Yoshizawa et al. 1997, Dardenne et al. 2001, Panda et al. 2001) as seen in human vitamin D-dependent rickets type II and I. It has recently been ascertained that bone mineralization is directly regulated by serum calcium whereas bone formation and remodeling are regulated by 1α,25-$(OH)_2D_3$/VDR system (Panda et al. 2004). Some biomarkers of bone formation, such as osteocalcin (Kerner et al. 1989) and alkaline phosphatase (Kyeyune-Nyombi et al. 1991) are induced by 1α,25-$(OH)_2D_3$. Some biomarkers of bone resorption may also be regulated. For

example, osteopontin (bone sialoprotein I) (Noda et al. 1990) is up-regulated and type I collagen (Harrison et al. 1989) is down-regulated by 1α,25-$(OH)_2D_3$.

In addition to the crucial role of 1α,25-$(OH)_2D_3$ in calcium homeostasis and bone growth, it has many other roles. It increases calcium uptake in myoblast cultures (Giuliani and Boland 1984) and cardiac muscle cells (Walters et al. 1987) at physiological concentrations. It regulates skin cell growth and differentiation (Gniadecki 1997, Gurlek et al. 2002) as well as the function of the immune system (Bhalla et al. 1984, Penna and Adorini 2000, Canning et al. 2001, Griffin et al. 2001) at physiological or pharmacological concentrations. At pharmacological concentrations it increases insulin synthesis and secretion (Bourlon et al. 1999) and regulates proliferation and differentiation of various cancer cells (Mehta and Mehta 2002, Lin and White 2004).

2.2. 1α,25-Dihydroxyvitamin D_3-mediated autocrine/paracrine system

Since the discovery of extra-renal 1α-hydroxylase, 1α,25-$(OH)_2D_3$-mediated autocrine/paracrine system has been proposed. It is believed that the extra-renal 1α,25-$(OH)_2D_3$ production acts as an antiproliferative or immunomodulatory steroid in the producing cells or the cells nearby and does not affect the systemic calcium balance. For example, in antigen presenting cells such as macrophages (Kreutz et al. 1993) and dendritic cells (Fritsche et al. 2003, Hewison et al. 2003), terminal differentiation increases 1α-hydroxylase expression and 1α,25-$(OH)_2D_3$ production, which thereafter, suppresses the differentiation and antigen-presentation capabilities of the precursor cells in a paracrine pathway. Meanwhile, terminal differentiation decreases VDR expression in the mature cells, which leads to the decrease in 1α,25-$(OH)_2D_3$-responsiveness. 1α-Hydroxylase expression in the parathyroid gland (Segersten et al. 2002), placenta and deciduas (Zehnder et al. 2002b), endothelial cells (Zehnder et al. 2002a), prostate epithelial cells (Barreto et al. 2000), and colon (Tangpricha et al. 2001) implies that local 1α,25-$(OH)_2D_3$ production may influence the proliferation and differentiation or immune function of those cells. Non-systemic effect of the locally produced 1α,25-$(OH)_2D_3$ depends largely on the autocrine induction of 24-hydroxylase (Hewison et al. 2004).

3. VITAMIN D_3 AND CANCERS

3.1. Vitamin D_3 action in cancers

1α,25-$(OH)_2D_3$ has potent antiproliferative, prodifferentiative, immunomodulatory, and apoptotic activities (Colston and Hansen 2002, Mehta and Mehta 2002, Ylikomi et al. 2002, Lamprecht and Lipkin 2003, Bikle 2004, Nagpal et al. 2005). 1α,25-$(OH)_2D_3$ and its analogs induce G1 growth arrest accompanied by either increased expression of cyclin-dependent kinase inhibitors $p21^{waf1}$ and $p27^{kip1}$, transforming growth factor-β1 (TGFβ1), and insulin-like growth factor binding protein-3 (IGFBP-3) or decreased expression of c-myc and phosphorylation of retinoblastoma protein in many types of cancer cells, such as prostate (Campbell et al. 1997, Zhuang and Burnstein 1998, Yang and Burnstein 2003), breast (Verlinden et al. 1998, Jensen et al. 2001), colon (Scaglione-Sewell et al. 2000), ovarian (Li et al. 2004), leukemia (Wang et al. 1996, Muto et al. 1999), and myeloma (Puthier et al. 1996). However, unlike in other tumors, 1α,25-$(OH)_2D_3$ decreases $p21^{waf1}$ expression in squamous cell carcinoma (Hershberger et al. 1999), which may reflect the differentiating effect of 1α,25-$(OH)_2D_3$ because $p21^{waf1}$ has inhibitory function in differentiation of keratinocytes (Di Cunto et al. 1998).

1α,25-$(OH)_2D_3$ or its analog induces cell differentiation by increasing E-cadherin (Palmer et al. 2001) and calcium sensing receptor (Chakrabarty et al. 2005) in colon cancer cells, involucrin (Gniadecki 1997) and phospholipase C-γ1 (Xie and Bikle 2001) in keratinocytes as well as prostate specific antigen (PSA) (Skowronski et al. 1993) and E-cadherin (Campbell et al. 1997) in prostate cancer cells.

1α,25-$(OH)_2D_3$ and its analogs up-regulate apoptotic markers cathepsin B and clusterin in breast cancer cells (Simboli-Campbell et al. 1996) and a proapoptotic protein Bak in colorectal carcinoma cells (Diaz et al. 2000a) and down-regulate an antiapoptotic protein Bcl-2 in breast cancer cells (James et al. 1998), prostate cancer cells (Crescioli et al. 2002, Guzey et al. 2002), and leukemia cells (Pepper et al. 2003).

1α,25-$(OH)_2D_3$ may also modulate the immune function (Lemire 1997, Lemire 2000). The nuclear factor-κB protein RelB (Dong et al. 2003), interleukin-2 (Bemiss et al. 2002), interleukin-12 (Lyakh et al. 2005), interferon-γ (Cippitelli and Santoni 1998), and tumor necrosis factor receptor 1 (Mathiasen et al. 2001) are targets of 1α,25-$(OH)_2D_3$ in the immune system. Despite the potent anticancer activities, 1α,25-$(OH)_2D_3$ has not been used in cancer prevention or treatment mainly because it causes severe hypercalcemia at the concentration that is effective in preventing experimental carcinogenesis or inhibiting cancer growth in experimental models.

3.2. Prostate cancer

3.2.1. Prostate cancer epidemiology

Prostate cancer is the third most common cancer in men, the most common cancer in Western countries, and the second most fatal cancer in American men (Chen and Holick 2003, Gronberg 2003). The cause of prostate cancer seems to be related to ethnic origin, age, family history, diet, environment, and hormones (Gronberg 2003, Bostwick et al. 2004). Three independent cohort studies showed a strong association between high serum levels of insulin-like growth factor-I and increased prostate cancer risk (Chan et al. 1998, Harman et al. 2000, Stattin et al. 2000). High level of circulating testosterone is not associated with increased prostate cancer risk (Hsing and Comstock 1993, Chen et al. 2003a, Stattin et al. 2004) and by contrast, both testosterone and DHT levels are lower in men with prostate cancer, especially, with more advanced tumors (Gustafsson et al. 1996, Hoffman et al. 2000). The initial hypothesis that vitamin D deficiency may be a risk factor for prostate was raised by Schwartz and Hulka (1990). Later some epidemiological studies showed a negative correlation between prostate cancer mortality and UV radiation exposure (Hanchette and Schwartz 1992, Luscombe et al. 2001, Freedman et al. 2002, Grant 2002) and further, an association between low levels of serum $25OHD_3$ and a higher risk of prostate cancer (Corder et al. 1993, Ahonen et al. 2000a). These data may offer an explanation for the increased risk of African American men and aged men to develop prostate cancer as a result of decreased synthesis of vitamin D_3. However, some studies showed no association between serum vitamin D_3 metabolites and prostate cancer (Braun et al. 1995, Gann et al. 1996, Nomura et al. 1998). A recent report showed that both low and high levels of serum $25OHD_3$ are associated with a higher risk of prostate cancer (Tuohimaa et al. 2004), which may be due to vitamin D_3 deficiency and resistance.

3.2.2. Sex steroid hormones in prostate development and carcinogenesis

The normal prostate consists of epithelial glands and fibromuscular stroma and the glands are well organized. In the prostate epithelium, there are three types of cells, secretory luminal cells, which are androgen-dependent and secrete PSA, basal cells, and neuroendocrine cells. In the prostate stroma, there are fibroblasts, smooth muscle cells, endothelial cells, nerves, dendritic cells, and lymphocytes. Because prostate carcinoma is derived from the epithelium, the cancer epithelial cells have been most studied. It is known that both epithelial and mesenchymal components are required for prostate differentiation and that androgens act first on the mesenchyme, and then on the epithelium to form the prostate (Abate-Shen and Shen 2000). Additionally, the prostate stroma plays an important role in carcinogenesis and aberrant interaction between stroma and epithelium is thought to contribute to carcinoma progression (Tlsty and Hein 2001, Chung et al. 2003, Bhowmick et al. 2004, Mueller and Fusenig 2004). Studies have shown that carcinoma-associated fibroblasts do not form tumor but promote carcinogenesis of non-tumorigenic prostate epithelial cells (Grossfeld et al. 1998, Olumi et al. 1999, Cunha et al. 2002). Therefore, both stroma and epithelium should be considered in the treatment of prostate cancer.

The prostate is an androgen-dependent organ. The male sex hormone testosterone is converted by 5α-reductase type II in the prostate, yielding 5α-dihydrotestosterone (DHT), which has 5-fold higher affinity for the androgen receptor (AR) than does testosterone (Feldman and Feldman 2001). AR is normally associated with heat shock proteins in cytoplasm and is dissociated upon ligand binding, which is followed by receptor phosphorylation, conformational changes, dimerization, and binding of androgen-responsive elements (Ruijter et al. 1999). It has been recognized for centuries that castration prevents growth of the prostate (Machtens et al. 2000) and therefore, androgen ablation therapy (surgical castration, administration of androgen antagonists, or 5α-reductase inhibitors) has been used in prostate cancer treatment. However, the tumor cells eventually become more advanced androgen-independent through several mechanisms, such as AR gene amplification and overexpression (Visakorpi et al. 1995, Koivisto et al. 1997, Linja et al. 2001). *In vitro* studies have shown that DHT has a biphasic effect on the growth of prostate cancer cells. It stimulates the growth of LNCaP cells at low concentrations (0.001 to 0.1 nM) and inhibits it at high concentrations (1 to 100 nM) (Sonnenschein et al. 1989, Henttu and Vihko 1992, Henttu et al. 1992, Lee et al. 1995, Zhao et al. 1997).

On the other hand, testosterone can be converted into estrogens by aromatase (Schweikert et al. 1976). Aromatase mRNA expression and/or enzyme activity were detected in the stroma of benign and malignant prostate tissues as well as in cancer cells, but not in nonmalignant prostate epithelial cells (Hiramatsu et al. 1997, Negri-Cesi et al. 1998, Negri-Cesi et al. 1999, Ellem et al. 2004). The two ER subtypes, ERα and β, have been found in the prostate (Royuela et al. 2001, Linja et al. 2003). The effects of estrogens in the prostate was earlier thought to be mainly in the prostate stromal compartment (Ekman 2000), because ERα was localized in prostate stroma. Later, ERβ was found to be expressed in the prostate epithelial cells and it has antiproliferative effects on the prostate (Weihua et al. 2002). Exogenous estrogens inhibit prostate growth by indirect effects caused by suppression of pituitary gonadotropins and testicular testosterone output (Harkonen and Makela 2004). The direct effects of estrogens on the prostate have been investigated using organ cultures of rat or human prostate. High doses of estrogens were shown to be growth inhibitory in the organ culture of rat ventral prostate (Jarred et al. 2000). In the

organ cultures of human prostate and rat dorsal-lateral prostate, estrogens were found to stimulate DNA synthesis and induce squamous metaplasia (Nevalainen et al. 1991, Nevalainen et al. 1993).

It has been suggested that the development of prostate cancer depends on both androgenic and estrogenic responses and that neither hormone alone can induce malignant changes on the prostate (Risbridger et al. 2003). This hypothesis is supported by the studies performed in the hypogonadal mice and aromatase knockout mice. A direct proliferative response to estrogens has been found in the hypogonadal mice, which are deficient in gonadotrophins and androgens. However, there was no evidence of malignant changes in the prostate of the hypogonadal mice (Bianco et al. 2002). The aromatase knockout mice had no estrogen production, but very high androgen concentration (McPherson et al. 2001). Those mice developed benign prostatic hyperplasia. However, no malignant changes were detected in the prostate of the aromatase knockout mice (McPherson et al. 2001).

3.2.3. Retinoic acid in prostate cancer

Retinoic acid is a derivative of vitamin A. ATRA is the main signaling retinoid in the body and exerts its action by binding to RARα, β, and γ. RARα and γ are expressed in both normal and malignant prostate tissues whereas the expression of RARβ is significantly reduced in malignant prostates (Kikugawa et al. 2000, Lotan et al. 2000). RARβ, as a tumor suppressor gene in lung and breast cancers (Houle et al. 1993, Sirchia et al. 2002), has been found to be hypermethylated in prostate cancer (Nakayama et al. 2001, Jeronimo et al. 2004). Both inhibitory and stimulatory effects of ATRA on prostate cell growth have been demonstrated (Fong et al. 1993, Peehl et al. 1993, Jones et al. 1997). Interestingly, some antagonists of RARs have been found to inhibit the growth of prostate cancer cells (Hammond et al. 2001, Keedwell et al. 2004). ATRA was found to induce differentiation of prostate epithelial cells, as measured by PSA secretion (Fong et al. 1993, Esquenet et al. 1996) and by the expression of cytokeratins 8 and 18 (Peehl et al. 1994). ATRA may promote apoptosis of prostate cancer cells (Huss et al. 2004) and potentiate Taxotere-induced cell death in prostate cancer (Wang and Wieder 2004). ATRA also has antimetastatic activity (Nwankwo 2002). ATRA, like 1α,25-$(OH)_2D_3$, also increases the expression of IGFBP-3 in LNCaP cells (Goossens et al. 1999). However, several clinical trials have shown that the antitumor effect of ATRA in patients with prostate cancer was modest or minimal (Culine et al. 1999, Kelly et al. 2000).

3.3. Vitamin D_3 and its metabolism in prostate cancer

3.3.1. Vitamin D_3 in prostate cancer

VDR is expressed in prostate cancer cells, such as LNCaP, PC3, and DU145 (Miller et al. 1992, Skowronski et al. 1993) as well as in primary stromal and epithelial cells derived from normal and malignant prostate tissues (Skowronski et al. 1995). It is clear that VDR is required in the antiproliferative action of 1α,25-$(OH)_2D_3$ (Hedlund et al. 1996a, Hedlund et al. 1996b), but VDR abundance does not correlate with differential antiproliferative activities of 1α,25-$(OH)_2D_3$ in various cancer cells (Zhuang et al. 1997). 1α,25-$(OH)_2D_3$ acts as an anticancer agent through different pathways in different prostate cell types. In androgen-sensitive prostate cancer cells LNCaP, 1α,25-$(OH)_2D_3$ inhibits cell proliferation (Skowronski et al. 1993, Skowronski et al. 1995), induces the secretion of PSA (Skowronski et al. 1993, Skowronski et al. 1995), and down-regulates the expression of proliferating cell nuclear antigen (Hsieh et al. 1996). It is

known that the antiproliferative effect of 1α,25-$(OH)_2D_3$ in LNCaP cells involves G1 growth arrest (Zhuang and Burnstein 1998), hypophosphorylation of retinoblastoma protein (Zhuang and Burnstein 1998), induction of IGFBP-3 and $p21^{waf1}$ (Boyle et al. 2001), stabilization of $p27^{kip1}$, and cyclin-dependent kinase 2 mislocalization (Yang and Burnstein 2003). 1α,25-$(OH)_2D_3$ also causes apoptosis in LNCaP cells (Hsieh and Wu 1997), accompanied by down-regulation of Bcl-2 (Blutt et al. 2000a). The action of 1α,25-$(OH)_2D_3$ is androgen-dependent in LNCaP cells (Zhao et al. 1997) and CWR22R cells (Bao et al. 2004) and is androgen-independent in MDA cells (Zhao et al. 2000). 1α,25-$(OH)_2D_3$ inhibits PC3 cell growth through induction of both TGFβ and IGFBP-3 production, and the TGFβ pathway seems to be essential (Murthy and Weigel 2004). Unlike in LNCaP cells, G1 accumulation is not observed in PC3 cells after 1α,25-$(OH)_2D_3$ treatment (Zhuang and Burnstein 1998). Additionally, 1α,25-$(OH)_2D_3$ inhibits the invasiveness of DU145 cells (Schwartz et al. 1997) and decreases cell adhesion of PC3 and DU145 (Sung and Feldman 2000).

Studies in animals showed that 1α,25-$(OH)_2D_3$ and its analogs significantly reduced prostate tumor volume (Schwartz et al. 1995a), tumor growth (Blutt et al. 2000b, Oades et al. 2002), and metastasis (Getzenberg et al. 1997, Lokeshwar et al. 1999). A few clinical trials of 1α,25-$(OH)_2D_3$ have been conducted in prostate cancer patients whose PSA level was significantly decreased (Osborn et al. 1995, Gross et al. 1998). However, all *in vivo* studies show that 1α,25-$(OH)_2D_3$ causes severe hypercalcemia and/or hypercalciuria. Therefore, the clinical use of 1α,25-$(OH)_2D_3$ is limited and lesser calcemic analogs would be promising.

3.3.2. Vitamin D_3 metabolism in prostate cancer

24-Hydroxylase is ubiquitous in the body and is an important enzyme controlling the action of vitamin D_3 metabolites. It has been shown that 1α,25-$(OH)_2D_3$ can induce the expression of 24-hydroxylase in many prostate cancer cells (Skowronski et al. 1993, Miller et al. 1995, Skowronski et al. 1995). Cells expressing high basal or induced level of 24-hydroxylase may be vitamin D_3 resistant. For example, DU145 cells, expressing a high level of 24-hydroxylase, are not inhibited by 1α,25-$(OH)_2D_3$ unless in the presence of 24-hydroxylase enzyme inhibitor (Ly et al. 1999). The antiproliferative activity of 1α,25-$(OH)_2D_3$ seems to be related in inverse proportion to 24-hydroxylase expression (Miller et al. 1995). Ketoconazole, a 24-hydroxylase inhibitor, has been proposed for use in combination with 1α,25-$(OH)_2D_3$ in prostate cancer treatment (Peehl et al. 2001, Peehl et al. 2002). The chromosomal region where the 24-hydroxylase gene is located has been found to be amplified in prostate cancer (Wolter et al. 2002), which may cause overexpression of 24-hydroxylase and abrogate vitamin D_3-mediate growth control. 24-Hydroxylase should, therefore, be considered in the use of vitamin D in prostate cancer treatment.

The autocrine/paracrine role of 1α,25-$(OH)_2D_3$ in the prostate was evidenced by the finding of 1α-hydroxylase activity in prostate cells (Schwartz et al. 1998). PC3, DU145, and primary prostate epithelial cells possess 1α-hydroxylase activity and no measurable production of 1α,25-$(OH)_2D_3$ was detected in LNCaP cells (Schwartz et al. 1998). $25OHD_3$ inhibits the proliferation of 1α-hydroxylase-possessing cells, which was assumed to be due to the conversion of $25OHD_3$ to 1α,25-$(OH)_2D_3$ (Barreto et al. 2000). Moreover, the less 1α-hydroxylase activity the prostate

cancer cells possess, the less sensitive they are to $25OHD_3$ (Hsu et al. 2001). Accordingly, $25OHD_3$ and its analogs have been evaluated as therapeutic agents for prostate cancer (Chen et al. 2000b, Swamy et al. 2004). Because of the less calcemic property, $25OHD_3$ and its analogs would be better anticancer agents than $1\alpha,25\text{-}(OH)_2D_3$. The regulation of prostate 1α-hydroxylase is different from that of renal 1α-hydroxylase. $1\alpha,25\text{-}(OH)_2D_3$, but not PTH or calcium, suppresses 1α-hydroxylase gene-promoter and enzyme activity in prostate epithelial cells (Young et al. 2004). It is possible that the activation of $25OHD_3$ by 1α-hydroxylase in the prostate may contribute to the antiproliferative activity of $25OHD_3$, but $25OHD_3$ itself may also have an effect on cell proliferation.

AIMS OF THE PRESENT STUDY

The aims of the present study were:

1. To study the expression and $25OHD_3$- and $1\alpha,25\text{-}(OH)_2D_3$-mediated regulation of two key enzymes involved in vitamin D_3 metabolism, namely 1α-hydroxylase and 24-hydroxylase, in prostate stromal and epithelial cells (I, II, III, IV)
2. To study the role of DHT in the transcriptional and antiproliferative activities of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ in prostate cancer epithelial cells (II, IV)
3. To study the combined effect of ATRA and vitamin D_3 metabolites on 24-hydroxylase expression in prostate stromal and epithelial cells (III)
4. To evaluate the hormonal role of $25OHD_3$ in prostate stromal and cancer cells (I, IV)

MATERIALS AND METHODS

1. MATERIALS (I, II, III, IV)

$25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ were generously donated by Leo Pharmaceuticals (Ballerup, Denmark). VID400, an inhibitor of 24-hydroxylase, and SDZ88-357, an inhibitor of 1α-hydroxylase, were kindly provided by Dr. Anton Stuetz (Novartis Research Institute, Vienna, Austria). LE135 and Am80 were kindly provided by Dr. Hiroyuki Kagechika (School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan) and BMS453 by Dr. Hinrich Gronemeyer (IGBMC, Department of Cell Biology and Signal Transduction, Illkirch, France). DHT was obtained from Merck (Darmstadt, Germany), Casodex from AstraZeneca (London, UK), and hydroxyflutamide from Schering-Plough Avondale (Rathdrum, Co. Wicklow, Ireland). All-trans-retinoic acid (ATRA) was obtained from ICN Biomedicals (Eschwege, Germany). Cycloheximide was purchased from Sigma (St. Louis, MO), TRIzol Reagent from Gibco BRL (Life Technologies, Grand Island, New York), and Lipofectamine™ 2000 from Invitrogen (Paisley, UK). RPMI-1640 and DMEM-F12 media were purchased from Sigma-Aldrich (Steinheim, Germany), FBS and penicillin-streptomycin from Gibco BRL (Groningen, The Netherlands), Amphothericin B and insulin from Sigma-Aldrich (St. Louis, MO, USA), BSA from Roche (Mannheim, Germany), and prostate epithelial cell growth medium from Cambrex (Walkersville, MD, USA). The expression vector pARL was kind gift from Dr. Albert O. Brinkmann (Department of Reproduction & Development, University Medical Center Rotterdam, The Netherlands) and pSG5-hRARβ from Dr. Hinrich Gronemeyer, (IGBMC, Department of Cell Biology and Signal Transduction, Illkirch, France).

2. CELL AND PRIMARY CULTURES (I, II, III, IV)

2.1. Cell cultures

Human prostate cancer cells LNCaP clone FGC, PC3, and DU145 were purchased from the American Type Culture Collection. Cells were routinely maintained in 75 cm^2 flasks with phenol red-free RPMI-1640 medium, supplemented with 10 % FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5 % CO_2 in air. To deplete endogenous steroids, the medium was changed to one containing 10 % DCC-FBS two to three days before starting the experiments.

2.2. Primary epithelial culture

The primary culture of normal human prostate epithelial cells PrEC was obtained from Cambrex (Walkersville, MD, USA). According to the manufacture's instructions, PrEC cells were cultured in prostate epithelial cell growth medium containing bovine pituitary extract, hydrocortisone, recombinant human epidermal growth factor, bovine insulin, epinephrine, triiodothyronine, transferrin, and gentamicin sulfate amphotericin-B (Cambrex, Walkersville, MD, USA). 1 % BSA was used in treatments of PrEC cells due to the lack of serum in the culture medium. The cells used in the experiments were from passage 6.

2.3. Primary stromal cultures

2.3.1. Tissues

Two primary cultures, designated P29SN and P32S, were derived from a benign area of prostate carcinoma and benign adenoma, respectively. The use of prostate tissue was approved by the local ethical committee and informed consent was obtained from both subjects.

2.3.2. Isolation and culture of primary stromal cells

Stromal cell cultures were established essentially according to previously described methods (Peehl and Sellers 1997). Tissue samples were minced into fragments not larger than 3 mm^3 and dissociated by enzymes. After overnight digestion at 37°C with 0.05 % collagenase A (P32S) or 0.05 % collagenase/dispase (P29SN), the partly digested tissue was centrifuged and digestion of the pellet was continued with fresh 0.1 % collagenase A at 37°C until isolated glands could be observed. Epithelial acini were separated from the stromal fraction by centrifugation at 50 g. The stromal fraction was carefully rinsed with culture medium and transferred to a 75 cm^2 culture flask. The primary stromal cells and serial cultures were maintained in phenol red-free DMEM/F12 medium, supplemented with 5 % DCC-FBS, 5 µg/ml insulin, and antibiotics (penicillin 100 units/ml, streptomycin 100 µg/ml, amphotericin B 2.5 µg/ml) at 37°C in humidified atmosphere of 5 % CO_2 in air. The cells used in the experiments were from passages 6 to 12.

3. TRANSIENT TRANSFECTIONS (II, III)

3.1. Transfection of AR

PC3 cells (6 x 10^5) were transfected with a wild-type human AR cDNA expression vector (pSG5-hAR) or the mutant AR of LNCaP cells (pARL) in 25 cm^2 flasks. Each flask received 1 µg of DNA and 2.5 µl of Lipofectamine™ 2000 in 1.5 ml of serum-free RPMI-1640 medium for 4 h following the manufacturer's instructions. After transfection, the cells were incubated in RPMI-1640 medium with 10 % DCC-FBS for 44 h to allow the expression of the transfected AR.

3.2. Transfection of RARβ

In the transfection of a human RARβ cDNA expression vector (pSG5-hRARβ) or the empty vector (pSG5), 8 x 10^4 of PC3 cells grown in 6-well plates were used with 100 ng of DNA and 0.3 µl of Lipofectamine™ 2000 in 1 ml of serum-free RPMI-1640 medium following the same procedure mentioned above in section 3.1.

4. CELL TREATMENTS AND RNA ISOLATION (I, II, III, IV)

The subconfluent cells were treated with vehicle (ethanol, final concentration 0.02-0.12 %), hormones, or other compounds at the concentrations indicated for 6, 24, and 48 h. The ethanol concentration was equal in controls and hormone-treated samples.

Total cellular RNA was isolated by using TRIzol Reagent following the manufacturer's instructions. Total RNA amounts were quantified by measuring absorbance at 260 nm. The OD_{260}/OD_{280} nm absorption ratio was always greater than 1.9. Denaturing agarose gel electrophoresis was performed to verify the integrity of RNA. The intensity of the 28S rRNA band was more than twice that of the 18S rRNA band stained by ethidium bromide.

5. REAL-TIME RT-PCR (I, II, III, IV)

5.1. Primer design

As recommended in the manufacturer's protocol, primers were designed using Primer Express v2.0 software (Perkin-Elmer Applied Biosystems, USA) to ensure suitability for the ABI Prism 7000 sequence detection system and the reaction parameters. To confirm the specificity of the

primer sequences, BLASTN searches were performed. All primers were designed to be intron-spanning to preclude amplification of genomic DNA. In order to normalize the amount of sample cDNA added to the reaction, human acidic ribosomal phosphoprotein P0 (RPLP0) was used as the endogenous control. RPLP0 is ubiquitously expressed and is considered to be a reliable endogenous control. All sequence specific oligonucleotide primers (Table 2) were synthesized by TAG Copenhagen A/S (Copenhagen, Denmark).

Table 2. Oligonucleotide primer sequences

Genes	**Oligonucleotide**	**Sequence**	**Position**
CYP24 (NM_000782)	forward primer	5'-GCCCAGCCGGGAACTC-3'	1907-1922
	reverse primer	5'-AAATACCACCATCTGAGGCGTATT-3'	1968-1945
CYP27B1 (NM_000785)	forward primer	5'-TTGGCAAGCGCAGCTGTAT-3'	1409-1427
	reverse primer	5'-TGTGTTAGGATCTGGGCCAAA-3'	1484-1464
VDR (NM_000376)	forward primer	5'-CCTTCACCATGGACGACATG-3'	948-967
	reverse primer	5'-CGGCTTTGGTCACGTCACT-3'	1025-1007
AR (NM_000044)	forward primer	5'-TGTCAACTCCAGGATGCTCTACTT-3'	3386-3409
	reverse primer	5'-ATTCGGACACACTGGCTGTACA-3'	3478-3457
RPLP0 (NM_001002)	forward primer	5'-AATCTCCAGGGGCACCATT-3'	515-533
	reverse primer	5'-CGCTGGCTCCCACTTTGT-3'	588-571
RARα (X06538)	forward primer	5’-AGTACTGCCGACTGCAGAAGTG-3’	648-669
	reverse primer	5’-TGTTTCGGTCGTTTCTCACAGA-3’	695-716
RARβ (X07282)	forward primer	5’-CAAATCATCAGGGTACCACTATGG-3’	601-624
	reverse primer	5’-CTGAATACTTCTGCGGAAAAAGC-3’	651-673
RARγ (M24857)	forward primer	5’-TGCCGGCTACAGAAGTGCTT-3’	847-866
	reverse primer	5’-CTTCTTGTTCCGGTCATTTCG-3’	895-915
RXRα (X52773)	forward primer	5’-TCCTTGGAGGCCTACTGCAA-3’	1270-1289
	reverse primer	5’-GGCAGGCGGAGCAAGAG-3’	1343-1327
RXRβ (M84820)	forward primer	5’-AGCAGCAGGGACGGTTTG-3’	1559-1576
	reverse primer	5’-GATGCTCTAGACACTTAAGGCCAAT-3’	1612-1636
RXRγ (U38480)	forward primer	5’-TTTCCCGCAGGCTATGGA-3’	58-75
	reverse primer	5’-TGCTGATGGGCTCATGGAT-3’	102-120

5.2. cDNA synthesis and real-time PCR

The total RNA from each sample was reverse-transcribed using a high-capacity cDNA achieve kit (Perkin-Elmer Applied Biosystems, USA) following the manufacturer's instructions. The experimental protocol was following: 10 min at 25°C followed by 120 min reverse transcription at 37°C. All PCR reactions were performed in MicroAmp optical 96-well reaction plates using an SYBR Green Master Mix kit (Perkin-Elmer Applied Biosystems, USA) on an ABI Prism 7000 sequence detection system (Perkin-Elmer Applied Biosystems, USA). The thermal cycling conditions consisted of a 10 min polymerase activation/initial denaturation at 95°C and 45 cycles with a 95°C denaturation for 15 sec and a 60°C annealing/extension for 1 min. Detection of accumulated fluorescent products was performed at the end of the extension step of each cycle. To verify the specific products, dissociation curve analysis was carried out after 45 cycles.

Serial dilutions of cDNA from the cells treated with 10 nM 1α,25-$(OH)_2D_3$ for 24 h were made to generate the standard curves of endogenous control and target genes. The calibrator sample used in the data analysis was the untreated sample. The data were quantified by the standard curve method with ABI Prism SDS Data Analysis software. The relative expression level of the target gene was calculated using amplification efficiencies obtained from the standard curves and Ct values as previously described (Pfaffl 2001). Results are expressed as means (± SD) of two to five independent experiments performed in duplicate.

5.3. Statistical methods

Statistical significance was evaluated by Student's *t*-test, one-way ANOVA, and two-way ANOVA followed by Bonferroni post-tests (GraphPad Prism 4 software, San Diego, CA, USA). Differences of $P > 0.05$ were considered not significant (NS), and $P < 0.05$ significant (*), $P < 0.01$ (**) and $P < 0.001$ (***) highly significant.

6. WESTERN BLOT ANALYSIS (I, II)

6.1. Analysis of 1α-hydroxylase

The subconfluent cells were trypsinized and pelleted. Cell lysate protein was prepared using M-Per™ mammalian protein extraction reagent (Pierce, Rockford, IL, USA) following the manufacturer's instructions. Protein concentrations were measured using BCA protein assay kit (Pierce, Rockford, IL, USA). Cell lysate was subjected to SDS-PAGE using a 7.5 % gel. Protein bands were transferred to nitrocellulose transfer membranes (0.45 μm pore; Schleicher & Schuell, Germany). After blocking of nonspecific binding sites with 20 % non-fat milk in TBS containing 0.1 % Tween 20 (TBS-T) at room temperature for one hour, the membranes were incubated with anti-mouse 1α-hydroxylase antibody (The Binding Site Ltd. Birmingham, UK) (Bland et al. 1999) at a 1:500 dilution in TBS-T containing 0.1 % non-fat milk at 4°C overnight. After washing with TBS-T, the membranes were incubated with secondary antibody (horseradish peroxidase-conjugated; ZYMED, CA, USA) at a 1:4000 dilution in TBS-T containing 0.1 % non-fat milk at room temperature for one hour. The blots were detected by enhanced chemiluminescence reagents (ECL, UK) and exposed to x-ray film for 2 min. The control experiment included presaturation of the primary antibody with an excess of the immunizing peptide (mouse amino acid sequence 266 to 289: R-H-V-E-L-R-E-G-E-A-A-M-R-N-Q-G-K-P-E-E-D-M-P-S) (Zehnder et al. 1999).

6.2. Analysis of AR

For the study of AR, the cytosolic and nuclear proteins were extracted and Western blot analysis was performed as described previously (Ahonen et al. 2000b). Polyclonal rabbit antibody AR70 used in Western blot analysis was produced against synthetic peptides corresponding to the N-terminal amino acids 1 to 17 of human AR (Tahka et al. 1997).

7. IMMUNOHISTOCHEMISTRY (I)

7.1. Cells

For immunohistochemical analysis cells from each primary stromal culture were grown on 4-well glass slides (Lab-Tek II Chamber Slide, Nalge Nunc, Naperville IL) until subconfluent. The cells were then fixed with 2 % formaldehyde for 20 min at room temperature and thereafter permeabilized with pre-chilled (-20°C) 94 % ethanol for 10 min on ice.

7.2. Antibodies

Mouse monoclonal anti-human antibodies were used to immunohistochemically characterize the stromal primary cultures. Antibodies against vimentin (1:200), desmin (1:100), smooth muscle actin (1:100), cytokeratins 5/6 (1:100), and 18 (1:50) were purchased from Dako (Glostrup, Denmark). Antibody against cytokeratins 14 (1:200) was from Novocastra (Newcastle, UK) and those against fibronectin (1:50) and cytokeratin 8 (1:50) from Santa Cruz (Santa Cruz, California, USA). Rat monoclonal anti-VDR antibody (1:200) was from Neo Markers (Fremont, CA, USA). Controls included omission of the primary antibodies and staining with nonimmunized mouse IgG. Normal rat IgG (Santa Cruz, California, USA) was used as control of VDR staining.

7.3. Immunostaining

The staining using the primary antibodies mentioned above was performed with a broad spectrum Zymed Histostain-Plus kit (Zymed Laboratories, South San Francisco, CA, USA) with the following modifications to the manufacturer's instructions: primary antibodies were incubated overnight at 4°C and biotinylated second antibody 20 min RT. All washings were repeated three times, 5 min each.

8. CELL GROWTH ASSAYS (I, III, IV)

8.1. Crystal violet staining assay

P29SN and P32S cell growth was analyzed by crystal violet staining assay performed in 96-well culture plates seeding 1000 cells/well in 200 µl medium. The cells were allowed to attach for 24 h. Cells were then treated with 100, 250, 1000 nM of $25OHD_3$ or 10 nM of $1\alpha,25\text{-}(OH)_2D_3$. Both control and treated cells received ethanol vehicle at a concentration of 0.1 %. Media were changed and treatments were renewed every 48 h. Relative cell numbers were quantified at 0, 3, 5, 7, 9, and 11 days by using crystal violet assay (Kueng et al. 1989). Briefly, cells were fixed with 11 % glutaraldehyde, washed with deionized water, air-dried, stained with 0.1 % crystal violet, washed with deionized water, and air-dried. Then 10 % acetic acid was added and a Victor 1420 Multilabel Counter (Wallac, Turku, Finland) was used for the optical density measurements of extracts at a wavelength of 590 nm. Two separate experiments were done in which six determinations were used for each treatment. Statistical significance was evaluated by Student's *t*-test.

8.2. Cell number counting assay

P32S or P29SN cells seeded in 24-well plates at a density of 5758 cells per well in 1 ml medium or in 6-well plates at a density of 3 x 10^4 cells per well in 3 ml medium were treated with vehicle (0.1 % ethanol), 100 nM, 250 nM of $25OHD_3$, or 10 nM of $1\alpha,25\text{-}(OH)_2D_3$ and/or Am80 (RAR selective ligand) at the concentrations indicated. Media were changed and treatments were renewed every 48 h. At day 6 or 9, cells were trypsinized and pelleted. Cell numbers were counted in a Burker chamber (Assistent, Sondheim, Germany). The experiments were performed three to five times independently and the results are expressed as percent of control (mean ± SD). Statistical significance was evaluated by Student's *t*-test or one-way ANOVA followed by Bonferroni post-tests (GraphPad Prism 4 software, San Diego, CA, USA).

LNCaP cell growth was analyzed by counting cell numbers. LNCaP cells seeded in 6-well plates at a density of 5 x 10^4 cells per well in 3 ml RPMI-1640 medium containing 10 % FBS were allowed to attach for 48 h and then treated with vehicle (0.1 % ethanol), 100 nM, 500 nM of $25OHD_3$, 0.1 nM, 10 nM of $1\alpha,25\text{-}(OH)_2D_3$, and/or 1 nM of DHT. Media were changed and treatments were renewed every 48 h. At day 6, cells were trypsinized and pelleted. Cell numbers were counted in a Burker chamber (Assistent, Sondheim, Germany). The experiments were performed three times independently and the results are expressed as percent of control (mean ± SD). Statistical significance was determined by two-way ANOVA followed by Bonferroni post-tests (GraphPad Prism 4 software, San Diego, CA, USA). Differences of $P > 0.05$ were considered not significant (NS), and $P < 0.05$ significant (*), $P < 0.01$ (**) and $P < 0.001$ (***) highly significant.

9. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS (I, II)

9.1. 1α-Hydroxylase activity assay

P29SN cells were seeded in 25 cm^2 flasks in 3 ml of complete growth medium. After 48 h of incubation, the medium was replaced with fresh medium containing 250 nM $25OHD_3$ and/or 1000 nM SDZ88-357. At 4 h, the media and cells were collected for quantitation of $1\alpha,25\text{-}(OH)_2D_3$ concentration. The radioactive 1α,25-dihydroxy (26,27 methyl-^{3}H) vitamin D_3 TRK 656 5 uCi, Amersham) was added and the samples were pre-purified using the acetonitrile-C18 Sep-Pak Cartridge (Waters, Ireland) (Turnbull et al. 1982) followed by separation of the metabolites by high-performance liquid chromatography (HPLC, Pharmacia LKB HPLC pump 2248, VWM 2141, Uppsala, Sweden). The mobile phase solvent system was hexane: dichloromethane: methanol: isopropanol (75:12:6:7). The concentrations of $1\alpha,25\text{-}(OH)_2D_3$ were quantified by radioreceptor assay (Reinhardt et al. 1984). The corresponding protein concentrations were measured using BCA protein assay kit (Pierce, Rockford, IL, USA). Enzymatic activity was expressed as fmol/mg protein/h. Data are expressed as means (± SD) of five repeats.

9.2. $1\alpha,25\text{-}(OH)_2D_3$ assay

LNCaP cells (3.5 X 10^5) cultured in 25 cm^2 flasks in the growth medium containing 10 % DCC-FBS for three days were pre-treated with 10 nM $1\alpha,25\text{-}(OH)_2D_3$ in the absence or presence of 10 nM DHT for 48 h and then incubated with a physiological concentration of $1\alpha,25\text{-}(OH)_2D_3$ for 16 h. The media and cells were collected for the assay of the unmetabolized $1\alpha,25\text{-}(OH)_2D_3$

following the procedure described above in section 9.1. Statistical significance was determined by Student's *t*-test.

RESULTS

1. REGULATION OF CELL GROWTH (I, III, IV)

1.1. Prostate stromal cell growth

1.1.1. Characterization of the primary stromal cultures

Both primary stromal cultures P29SN and P32S showed similar staining characteristics. An extensive staining for vimentin and fibronectin was seen, over 99 % of cells being positive for these markers. Less than 5 % of the cells present expressed smooth muscle actin and less than 2 % expressed desmin. There was no specific staining with anti-cytokeratins 8 and 18. Stainings with anti-cytokeratins 5/6 and 14 as well as PBS were negative. A positive immunostaining for VDR was detected in the discrete foci of cell nuclei. The control staining for VDR was negative. These data indicate that the vast majority of both primary prostate cultures were fibroblasts in phenotype.

1.1.2. Effect of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$

The crystal violet assays showed that the growth of P29SN cells was significantly inhibited when treated with 250 nM, 1000 nM of $25OHD_3$, and 10 nM of $1\alpha,25\text{-}(OH)_2D_3$. $25OHD_3$ dose-dependently inhibited P29SN cell growth. Compared to the controls, the relative cell growth at day 9 treated with 100 nM, 250 nM, 1000 nM of $25OHD_3$, and 10 nM of $1\alpha,25\text{-}(OH)_2D_3$ was 98 ± 25 % ($P > 0.05$), 70 ± 8 % ($P < 0.01$), 51 ± 6 % ($P < 0.0001$), and 62 ± 9 % ($P < 0.001$), respectively (Table 3). To verify the results above, a cell number counting method was applied. Compared to the controls, at day 9 the relative growth of P29SN cells treated with 100 nM, 250 nM of $25OHD_3$, and 10 nM of $1\alpha,25\text{-}(OH)_2D_3$ was 139 ± 16% ($P = 0.050$), 78 ± 5% ($P = 0.018$), and 68 ± 8% ($P = 0.023$), respectively.

The crystal violet assays showed that the growth of P32S cells was not inhibited by 10 nM of $1\alpha,25\text{-}(OH)_2D_3$ but significantly inhibited by $25OHD_3$. Compared to the controls, at day 9 the relative cell growth treated with 100 nM, 250 nM, 1000 nM of $25OHD_3$, and 10 nM of $1\alpha,25\text{-}(OH)_2D_3$ was 81 ± 12 % ($P < 0.01$), 77 ± 9 % ($P < 0.01$), 60 ± 7 % ($P < 0.0001$), and 95 ± 20 % ($P > 0.05$), respectively (Table 3).

Table 3. Antiproliferative effect of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ on prostate stromal cells (relative cell growth at day 9: percent of control, mean ± SD, n = 12)

	$25OHD_3$ (nM)				$1\alpha,25\text{-}(OH)_2D_3$ (nM)
	0	100	250	1000	10
P29SN cells	100 %	98±25 %	70±8 %	51±6 %	62±9 %
P32S cells	100 %	81±12 %	77±9 %	60±7 %	95±20 %

1.1.3. Effect of RAR-selective ligand Am80

Am80 at the concentrations of 1 to 10 nM had no effect on the growth of P32S cells, whereas at 100 and 200 nM concentration it strongly inhibited cell growth by 43.9% ($P < 0.01$) and 44.4%, respectively ($P < 0.001$).

1.1.4. Combined effect of Am80 and 1α,25-$(OH)_2D_3$ or 25OHD_3

The combined treatment of 1α,25-$(OH)_2D_3$ and Am80 at 10 nM concentration exhibited strong inhibitory effect ($P < 0.01$), whereas either of these alone had no effect on cell growth. Am80 at 1 or 100 nM, the same enhancement was observed but it was not statistically significant. Due to the strong inhibitory effect of 200 nM Am80, no enhanced effect of 1α,25-$(OH)_2D_3$ was observed. Moreover, 25OHD_3 inhibited the growth of P29SN cells ($P < 0.05$) and the effect was enhanced in the presence of 200 nM Am80 ($P < 0.001$).

1.2. Prostate cancer epithelial cell growth

1.2.1. Effect of 25OHD_3 and 1α,25-$(OH)_2D_3$

LNCaP cell growth was significantly inhibited by 25OHD_3 at 500 nM and by 1α,25-$(OH)_2D_3$ at a pharmacological concentration of 10 nM by 43% ($P < 0.001$) and 76% ($P < 0.001$), respectively (Table 4). However, 25OHD_3 at a physiological concentration of 100 nM and 1α,25-$(OH)_2D_3$ at a physiological concentration of 0.1 nM did not affect LNCaP cell growth ($P > 0.05$, Table 4).

1.2.2. Effect of DHT

DHT (1 nM) caused a 54% decrease in LNCaP cell growth ($P < 0.001$, Table 4).

1.2.3. Combined effect of DHT and 25OHD_3 or 1α,25-$(OH)_2D_3$

The combined treatment of 1 nM DHT with either 25OHD_3 or 1α,25-$(OH)_2D_3$ at physiological concentrations suppressed cell growth by 71% ($P < 0.001$) and by 72% ($P < 0.001$), respectively (Table 4). 1 nM DHT enhanced the effect of 500 nM 25OHD_3 from 43% inhibition to 90% inhibition ($P < 0.001$). The effect of 10 nM 1α,25-$(OH)_2D_3$ was increased from 76% inhibition to 89% inhibition by 1 nM DHT ($P > 0.05$, Table 4). It should be noted that the combined treatment of 1 nM DHT and either 25OHD_3 or 1α,25-$(OH)_2D_3$ was more antiproliferative than 1 nM DHT alone ($P < 0.05$; $P < 0.001$; $P < 0.01$). Two-way ANOVA shows a statistically significant interaction between two factors: 25OHD_3 and DHT ($F = 5.668$, $P = 0.0185$) and between 1α,25-$(OH)_2D_3$ and DHT ($F = 13.3$, $P = 0.0009$).

Table 4. Antiproliferative effect of 25OHD_3 and 1α,25-$(OH)_2D_3$ with and without DHT on LNCaP cells (relative cell growth at day 6: percent of control, mean ± SD, n = 3)

		25OHD_3 (nM)			1α,25-$(OH)_2D_3$ (nM)	
		0	100	500	0.1	10
DHT (nM)	0	100 %	101±9 %	57±7 %	100±21 %	24±8 %
	1	46±11 %	29±2 %	10±5 %	28±4 %	11±2 %

2. VDR EXPRESSION (II, III)

VDR was detected in all the cells studied. Neither 10 nM 1α,25-$(OH)_2D_3$ nor DHT at concentrations of 0.01 to 100 nM altered the expression of VDR mRNA in LNCaP cells. The level of VDR mRNA was not altered by ATRA in P29SN and PrEC cells.

3. RARs and RXRs (III)

3.1. Differential mRNA expression in epithelial and stromal cells

To better understand the action of ATRA, we studied the expression of RARs and RXRs. LNCaP cells expressed a significantly lower level of RARα than PrEC ($P < 0.05$). In the other cell types the expression level of the receptor did not differ. Of particular interest, RARβ was expressed differentially in prostate epithelial and stromal cells. The stromal cells expressed a much higher level of RARβ than the epithelial cells (LNCaP *vs.* P29SN, $P < 0.001$; LNCaP *vs.* P32S, $P < 0.001$; PrEC *vs.* P29SN, $P < 0.001$; PrEC *vs.* P32S, $P < 0.001$), while there was no significant difference between LNCaP and PrEC or between P29SN and P32S. The prostate cancer cells, LNCaP, expressed less RARγ than the other types of cells ($P < 0.001$) and PrEC cells expressed less RARγ than stromal cells (PrEC *vs.* P29SN, $P < 0.01$; PrEC *vs.* P32S, $P < 0.05$).

The expression of RXRα was slightly higher in epithelial than in stromal cells (PrEC *vs.* P29SN, $P < 0.05$; PrEC *vs.* P32S, $P < 0.01$; LNCaP *vs.* P32S, $P < 0.05$). RXRβ was similarly expressed in all cell types except for a significant difference between LNCaP and P29SN cells ($P < 0.05$). The expression of RXRγ was extremely low or negligible in P29SN, P32S, and PrEC cells.

3.2. Differential mRNA regulation by ATRA in epithelial and stromal cells

ATRA at 1000 nM did not regulate RARα and RARγ expression in any studied cell type. However, ATRA caused a 16-fold increase in RARβ mRNA level in PrEC cells ($P = 0.018$), a 3-fold increase in P29SN cells ($P = 0.031$), and a 5-fold increase in P32S cells ($P = 0.029$) but not in cancer LNCaP cells. The expression of RXRα mRNA appeared to be unaltered by 1000 nM ATRA in LNCaP, PrEC, and P29SN cells and slightly down-regulated 0.7-fold in P32S cells ($P = 0.043$). The expression of RXRβ mRNA was slightly decreased 0.6-fold in P29SN ($P = 0.027$) and increased 1.4-fold in P32S ($P = 0.002$), and further, unaltered in LNCaP and PrEC cells.

4. 1α-HYDROXYLASE EXPRESSION (I, IV)

4.1. In stromal cells

Immunoblotting analysis using an anti-mouse 1α-hydroxylase antibody showed a clear single band at 56 KD in both primary stromal cultures P29SN and P32S, which is the size of 1α-hydroxylase protein. No signal was seen in the presaturation controls.

To determine whether the primary prostate stromal cells can produce 1α,25-$(OH)_2D_3$, we performed a 1α-hydroxylase activity assay. 1α-Hydroxylase activity in P29SN cells was 30 ± 29 fmol/mg protein/h (n = 5). However, the concentration of 1α,25-$(OH)_2D_3$ in culture medium and in cells was much lower than physiological concentration (50 pM). When the cells received 250 nM $25OHD_3$ in the presence of 1000 nM SDZ88-357, a specific 1α-hydroxylase inhibitor (Schuster et al. 2001b), 1α-hydroxylase activity was 7 ± 36 fmol/mg protein/h (n = 5), which indicates that SDZ88-357 can effectively inhibit 1α-hydroxylase activity.

In both P29SN and P32S cells, quantitative real-time RT-PCR showed a detectable and similar level of 1α-hydroxylase mRNA. Among these, only in P29SN cells did the use of 100 nM

$25OHD_3$ for 6 h cause statistically significant up-regulation of 1α-hydroxylase mRNA level (2 ± 0.3-fold, $P < 0.05$).

4.2. In epithelial cells

Immunoblotting analysis using an anti-mouse 1α-hydroxylase antibody showed a weak band of 56 kD in DU145 cells. No signal was seen in the presaturation controls. However, in LNCaP cells, a very weak band of 56 kD was found along with a non-specific band at 64.6 kD, which did not disappear in the presaturation control. Quantitative real-time RT-PCR showed no regulation of 1α-hydroxylase expression by 500 nM $25OHD_3$, 10 nM 1α,25-$(OH)_2D_3$, or 1 nM DHT in LNCaP cells. The combined treatment of 500 nM $25OHD_3$ and 1 nM DHT caused a 1.8-fold increase in the level of 1α-hydroxylase mRNA ($P < 0.05$). However, the combined treatment of 10 nM 1α,25-$(OH)_2D_3$ and 1 nM DHT did not alter the level of 1α-hydroxylase mRNA.

Normal epithelial PrEC cells expressed a much higher basal 1α-hydroxylase mRNA level than stromal P29SN cells and cancer LNCaP, PC3, and DU145 cells ($P < 0.001$). The level of 1α-hydroxylase mRNA expressed in PrEC cells was 358-fold higher than that in LNCaP cells ($P < 0.001$). Further, P29SN cells expressed 0.8-fold less and PC3 and DU145 cells 17-fold and 21-fold more 1α-hydroxylase mRNA than LNCaP cells, respectively ($P > 0.05$).

5. 24-HYDROXYLASE EXPRESSION (I, II, III, IV)

5.1. mRNA expression and regulation in stromal cells

5.1.1. Effect of $25OHD_3$ and 1α,25-$(OH)_2D_3$

$25OHD_3$ and 1α,25-$(OH)_2D_3$ induced the expression of 24-hydroxylase mRNA in primary prostate stromal cells P29SN and P32S in a concentration- and time-dependent manner. $25OHD_3$ at a physiological concentration of 250 nM and 1α,25-$(OH)_2D_3$ at a pharmacological concentration of 10 nM were effective.

100 nM $25OHD_3$ at 6 h increased the mRNA level of 24-hydroxylase 2.27 ± 0.32-fold ($P > 0.05$) in P29SN cells and had no effect in P32S cells, while 250 nM and 1000 nM $25OHD_3$ enhanced the mRNA level of 24-hydroxylase in P29SN (200 ± 5-fold, $P < 0.01$ and 12000 ± 220-fold, $P < 0.01$, respectively) and in P32S cells (4 ± 0.5-fold, $P < 0.05$ and 660 ± 5-fold, $P < 0.0001$, respectively). In P29SN and P32S cells, treatment with 250 nM $25OHD_3$ caused a 140 ± 10-fold ($P < 0.05$) and 7 ± 2-fold ($P > 0.05$) stimulation at 24 h, a 90 ± 0.2-fold ($P < 0.01$) and 5 ± 0.7-fold ($P < 0.05$) stimulation at 48 h in 24-hydroxylase mRNA level, respectively.

1α,25-$(OH)_2D_3$ at a physiological concentration (0.1 nM) had no effect on the expression of 24-hydroxylase mRNA at 6 h in either primary culture. In P29SN cells, 10 nM 1α,25-$(OH)_2D_3$ increased 24-hydroxylase mRNA level 6900 ± 500-fold ($P < 0.001$), 14600 ± 800-fold ($P < 0.0001$), and 2900 ± 500-fold ($P < 0.01$) at 6, 24, and 48 h, respectively. Similarly, in P32S cells, 10 nM 1α,25-$(OH)_2D_3$ increased 24-hydroxylase mRNA level 4200 ± 1600-fold ($P > 0.05$), 34000 ± 200-fold ($P < 0.01$), and 18000 ± 200-fold ($P < 0.01$) at 6, 24, and 48 h, respectively.

5.1.2. Effect of 24-hydroxylase inhibitor, VID400

The transcriptional activity of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ was greatly increased in the presence of an inhibitor of 24-hydroxylase, VID400 (Schuster et al. 2001a, Schuster et al. 2001b). Combination of 250 nM $25OHD_3$ and 100 nM VID400 exhibited an 8.3-fold ($P < 0.05$) and 60-fold ($P < 0.05$) stimulatory effect compared to 250 nM $25OHD_3$ alone in P29SN and P32S cells, respectively. Moreover, 100 nM VID400 increased the 10 nM $1\alpha,25\text{-}(OH)_2D_3$-mediated induction of 24-hydroxylase mRNA 1.9-fold ($P < 0.05$) and 2.5-fold ($P > 0.05$) in P29SN and P32S cells, respectively.

5.1.3. Effect of 1α-hydroxylase inhibitor, SDZ88-357

By using a specific inhibitor for 1α-hydroxylase, SDZ88-357, which has been shown earlier to inhibit 1α-hydroxylase activity, we found that 250 nM $25OHD_3$ is capable of inducing 24-hydroxylase mRNA and 1α-hydroxylation is not a prerequisite for its hormonal activity of $25OHD_3$. In the presence of 1000 nM SDZ88-357, 250 nM $25OHD_3$ was 4 times ($P > 0.05$) and 2 times ($P > 0.05$) more effective than in the absence of 1α-hydroxylase inhibitor in P29SN and P32S cells, respectively. In the presence of both 100 nM VID400 and 1000 nM SDZ88-357, the induction of 24-hydroxylase mRNA by 250 nM $25OHD_3$ was increased 6-fold and 4-fold in P29SN and P32S cells, respectively.

5.1.4. Effect of ATRA and RAR-selective ligands

In P29SN cells, ATRA at 100 and 1000 nM did not affect the expression of 24-hydroxylase mRNA. However, ATRA significantly antagonized the effect of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$. The level of 24-hydroxylase mRNA induced by 250 nM $25OHD_3$ was decreased by 61 % ($P < 0.001$) with 100 nM ATRA and by 95 % ($P < 0.001$) with 1000 nM ATRA, respectively (Table 5). The level of 24-hydroxylase mRNA induced by 10 nM $1\alpha,25\text{-}(OH)_2D_3$ was decreased by 36 % ($P < 0.001$) with 100 nM ATRA and by 83 % ($P < 0.001$) with 1000 nM ATRA, respectively (Table 5). Two-way ANOVA shows a statistically significant interaction between the two hormones: $25OHD_3$ and ATRA ($F = 35.13$, $P < 0.0001$), $1\alpha,25\text{-}(OH)_2D_3$ and ATRA ($F = 42.20$, $P < 0.0001$).

In P32S cells, ATRA at 100 and 1000 nM had no significant effect on the expression of 24-hydroxylase mRNA. However, it significantly reduced the effect of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$. The level of 24-hydroxylase mRNA induced by 250 nM $25OHD_3$ was decreased by 38 % ($P < 0.001$) with 100 nM ATRA and by 96 % ($P < 0.001$) with 1000 nM ATRA, respectively (Table 5). The level of 24-hydroxylase mRNA induced by 10 nM $1\alpha,25\text{-}(OH)_2D_3$ was decreased by 89 % ($P < 0.001$) with 100 nM ATRA and by 98 % ($P < 0.001$) with 1000 nM ATRA, respectively (Table 5). Two-way ANOVA shows a statistically significant interaction between the two hormones: $25OHD_3$ and ATRA ($F = 184.56$, $P < 0.0001$), $1\alpha,25\text{-}(OH)_2D_3$ and ATRA ($F = 53.69$, $P < 0.0001$).

Table 5. Effect of ATRA on the $25OHD_3$- and $1\alpha,25\text{-}(OH)_2D_3$-mediated induction of 24-hydroxylase mRNA expression in prostate stromal cells (mean ± SD, n = 3)

	P29SN cells			P32S cells		
	ATRA (nM)			ATRA (nM)		
	0	100	1000	0	100	1000
Vehicle	1	1±0.6	0.8±0.2	1	3±2	3±2
$25OHD_3$ (250 nM)	31583±7531	12310 ±1513	1641±35	411±6	253±43	16±5
$1\alpha,25\text{-}(OH)_2D_3$ (10 nM)	34127±3714	21952 ±5378	5748±715	43595±8996	4919±3461	823±686

To further determine which retinoid receptor plays a role in the suppression of 24-hydroxylase expression in stromal cells, we applied two RAR-selective ligands, BMS453 and LE135. BMS453 binds with the same affinity to RARα, RARβ, and RARγ and it is a potent antagonist for RARα and RARγ, whereas it acts as a mixed agonist/antagonist for RARβ (Chen et al. 1995, Germain et al. 2004). LE135 does not bind to RXRs and RARγ, whereas it binds with higher affinity to RARβ (K_i = 0.22 μM) than to RARα (K_i = 1.4 μM) and acts as RARβ-selective antagonist (Li et al. 1999b).

We used BMS453 and LE135 at 1000 nM (Chen et al. 1995, Li et al. 1999b) in combination with 100 nM ATRA in P32S cells. The inhibitory effect of ATRA on $1\alpha,25\text{-}(OH)_2D_3$-induced expression of 24-hydroxylase mRNA was reversed by BMS453 ($P < 0.05$) and unaltered by LE135, indicating that ATRA acts through RARα and/or RARγ but not RARβ. In the study of RARβ mRNA expression, 100 nM ATRA induced the expression of RARβ 3.1 fold ($P < 0.001$), which was not affected by LE135. However, BMS453 reduced the ATRA-induced expression of RARβ by 29 % ($P < 0.01$), indicating that RARα and/or RARγ but not RARβ mediate(s) the ATRA action in the induction of RARβ.

To ascertain whether RARα or RARγ mediates the action of ATRA, we used an RARα agonist Am80. Am80 selectively activates RARα (K_i = 6.5 nM) and RARβ (K_i = 30 nM), and cannot bind to RARγ and RXRs (Umemiya et al. 1997). Am80 exhibited the same effect as ATRA. Am80 at 200 nM reduced the $1\alpha,25\text{-}(OH)_2D_3$-induced expression of 24-hydroxylase mRNA by 80.2% in P32S cells ($P < 0.001$). Moreover, 200 nM Am80 induced the expression of RARβ 3.8 fold in P32S cells ($P < 0.05$), which was as effective as 100 nM ATRA (4.0-fold induction, $P < 0.05$). Taken together, RARα mediates the action of ATRA in inhibiting the $1\alpha,25\text{-}(OH)_2D_3$-induced expression of 24-hydroxylase mRNA and in inducing the expression of RARβ in prostate stromal cells.

5.2. mRNA expression and regulation in primary epithelial cells

5.2.1. Effect of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$

In PrEC cells, 250 nM $25OHD_3$ and 10 nM $1\alpha,25\text{-}(OH)_2D_3$ at 24 h increased 24-hydroxylase mRNA level 317 ± 6-fold ($P < 0.001$) and 316 ± 33-fold ($P < 0.01$), respectively.

5.2.2. Effect of ATRA

In PrEC cells, ATRA (100 and 1000 nM) had no effect on the expression of CYP24 mRNA and did not affect the action of either $25OHD_3$ or $1\alpha,25\text{-}(OH)_2D_3$ (two-way ANOVA: $25OHD_3$ *vs.* ATRA, F = 2.14, $P = 0.1599$; $1\alpha,25\text{-}(OH)_2D_3$ *vs.* ATRA, F = 0.84, $P = 0.4561$).

5.3. Expression and regulation in cancer epithelial cells

5.3.1. Effect of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$

In LNCaP cells, both 10 nM $1\alpha,25\text{-}(OH)_2D_3$ and 1000 nM $25OHD_3$ time-dependently induced the expression of 24-hydroxylase mRNA. 10 nM $1\alpha,25\text{-}(OH)_2D_3$ increased 24-hydroxylase mRNA level 1218 ± 220-fold ($P > 0.05$), 4294 ± 16-fold ($P < 0.01$), and 2513 ± 118-fold ($P < 0.05$) at 6, 24, and 48 h, respectively. 1000 nM $25OHD_3$ increased 24-hydroxylase mRNA level 3730 ± 342-fold ($P < 0.05$), 11618 ± 199-fold ($P < 0.01$), and 9300 ± 906-fold ($P< 0.05$) at 6, 24, and 48 h, respectively. $25OHD_3$ at 500 nM and 750 nM also increased 24-hydroxylase mRNA level 77 ± 6-fold ($P > 0.05$), 21387 ± 1082-fold ($P < 0.01$), respectively. However, neither $1\alpha,25\text{-}(OH)_2D_3$ at a physiological concentration (0.1 nM) nor 100-250 nM $25OHD_3$ affected the expression of 24-hydroxylase mRNA at 24 h in LNCaP cells. The induction of 24-hydroxylase mRNA by $1\alpha,25\text{-}(OH)_2D_3$ requires protein synthesis, because a protein synthesis inhibitor, cycloheximide, strongly inhibited $1\alpha,25\text{-}(OH)_2D_3$-induction of 24-hydroxylase mRNA by 90 % ($P < 0.001$) in LNCaP cells.

24-Hydroxylase mRNA level in PC3 cells was increased 149 ± 29-fold ($P < 0.001$) and 288 ± 84-fold ($P < 0.05$) at 24 h by 500 nM $25OHD_3$ and 10 nM $1\alpha,25\text{-}(OH)_2D_3$, respectively. 10 nM $1\alpha,25\text{-}(OH)_2D_3$ increased 24-hydroxylase mRNA level 45 ± 11-fold ($P < 0.05$) in DU145 cells.

5.3.2. Effect of DHT

DHT alone at all concentrations tested had no significant effect on the 24-hydroxylase mRNA expression in LNCaP cells ($P > 0.05$, Table 6). The $1\alpha,25\text{-}(OH)_2D_3$-induced expression of 24-hydroxylase in LNCaP cells was strongly decreased by DHT at 1, 10, 100 nM by 50 % ($P < 0.001$), 94 % ($P < 0.001$), 97 % ($P < 0.001$), respectively, and slightly increased by DHT at 0.01 and 0.1 nM by 27 % ($P < 0.001$) and 37 % ($P < 0.001$), respectively (Table 6). Similarly, the $25OHD_3$-induced expression of 24-hydroxylase in LNCaP cells was strongly decreased by DHT at 1, 10, 100 nM by 75 % ($P < 0.05$), 97 % ($P < 0.01$), 99 % ($P < 0.001$), respectively, and slightly increased by DHT at 0.01 and 0.1 nM by 11 % ($P > 0.05$) and 3 % ($P > 0.05$), respectively (Table 6). This DHT effect is mediated by AR. Hydroxyflutamide (an agonist for the mutant AR of LNCaP cells) at 1 μM strongly inhibited the $1\alpha,25\text{-}(OH)_2D_3$-induced expression of 24-hydroxylase by 97 % ($P < 0.001$) and did not change the DHT effect. A pure antagonist, Casodex (1 μM), did not affect $1\alpha,25\text{-}(OH)_2D_3$-stimulated expression of 24-hydroxylase mRNA and partially antagonized the DHT effect. DHT did not significantly enhance the degradation of 24-hydroxylase mRNA. The biological consequence of the inhibitory effect of DHT on the expression of 24-hydroxylase mRNA is the protection of $1\alpha,25\text{-}(OH)_2D_3$ from catabolism. Pre-treatment with 10 nM $1\alpha,25\text{-}(OH)_2D_3$ for 48 h significantly enhanced the catabolism of $1\alpha,25\text{-}(OH)_2D_3$ ($P = 0.0312$ *vs.* no cell control and $P = 0.0270$ *vs.* one pre-treated with 10 nM $1\alpha,25\text{-}(OH)_2D_3$ and 10 nM DHT). However, pre-incubation with 10 nM $1\alpha,25\text{-}$

$(OH)_2D_3$ and 10 nM DHT did not cause a significant change in 1α,25-$(OH)_2D_3$ concentration, indicating that 10 nM DHT protects 1α,25-$(OH)_2D_3$ from catabolism.

Table 6. Effect of DHT on the $25OHD_3$- and 1α,25-$(OH)_2D_3$-mediated induction of 24-hydroxylase mRNA expression in LNCaP cells (mean ± SD, n = 3)

	DHT (nM)					
	0	0.01	0.1	1	10	100
Vehicle	1	10±15	3±2	6±4	11±11	2±1
$25OHD_3$ (500 nM)	69±5	77±29	71±4	17±23	2±2	0.8±0.5
1α,25-$(OH)_2D_3$ (10 nM)	30360±405	38489±748	41547±5929	15266±737	1771±220	1056±384

However, DHT does not affect the action of 1α,25-$(OH)_2D_3$ in inducing 24-hydroxylase in androgen-insensitive DU145 or PC3 cells whether transfected with the wild-type AR or the mutant AR of LNCaP cells. The levels of AR mRNA in PC3 and DU145 cells were, respectively, 0.32 % ($P < 0.0001$) and 0.051 % ($P < 0.0001$) of that in LNCaP cells. Two-way ANOVA shows no significant interaction between the two factors: 1α,25-$(OH)_2D_3$ and DHT ($P > 0.05$).

5.3.3. Effect of ATRA

The expression of 24-hydroxylase mRNA was not regulated by ATRA at 100 and 1000 nM in either LNCaP or PC3 cells. ATRA did not significantly interfere with either $25OHD_3$ or 1α,25-$(OH)_2D_3$ in regulating the expression of CYP24 mRNA in either LNCaP cells or PC3 cells whether transfected with RARβ or not.

DISCUSSION

1. LOCAL METABOLISM OF 25OHD$_3$ AND 1α,25-(OH)$_2$D$_3$ IN THE PROSTATE

This study shows evidence for the prostatic expression of two key enzymes involved in vitamin D$_3$ metabolism, 1α-hydroxylase and 24-hydroxylase. By using quantitative real-time RT-PCR and immunoblotting, we demonstrate, for the first time, the expression of 1α-hydroxylase in prostate stromal cells. 1α-Hydroxylase mRNA was not detected in prostate stromal cells in an early report (Barreto et al. 2000) possibly due to the low sensitivity of the traditional RT-PCR. Our results indicate that the stromal cells express more 1α-hydroxylase protein than cancer epithelial cells DU145 and LNCaP. However, the activity of this enzyme in stromal cells was low, being approximately 40-100-fold lower than that reported in normal epithelial cells (Hsu et al. 2001). Quantitative real-time RT-PCR showed that normal epithelial PrEC cells expressed a much higher basal level of 1α-hydroxylase mRNA than stromal P29SN cells and cancer LNCaP, PC3, and DU145 cells. Previous study showed that the promoter activity of 1α-hydroxylase gene was lower in PC3 and DU145 cells than that in normal prostate cells and was lost in LNCaP cells leading to the defect in 1α-hydroxylase enzyme activity (Chen et al. 2003c). Quantitative real-time RT-PCR showed that the expression of 1α-hydroxylase mRNA was not regulated by either 25OHD$_3$ or 1α,25-(OH)$_2$D$_3$ in LNCaP cells, but was up-regulated by 100 nM 25OHD$_3$ in P29SN cells. Earlier reports show that renal 1α-hydroxylase is down-regulated by 1α,25-(OH)$_2$D$_3$ at transcriptional level (Murayama et al. 1998, Kong et al. 1999) but the extra-renal expression of CYP27B1 mRNA is unaffected by 1α,25-(OH)$_2$D$_3$ in lung cancer cells (Jones et al. 1999), macrophages (Monkawa et al. 2000), and keratinocytes (Xie et al. 2002). It is possible that production of 1α,25-(OH)$_2$D$_3$ in the prostate acts as an autocrine/paracrine regulator. This 1α,25-(OH)$_2$D$_3$-mediated autocrine/paracrine system has been proposed earlier in the prostate (Barreto et al. 2000), breast (Townsend et al. 2005), colon (Bises et al. 2004), and immune system (Hewison et al. 2004).

The action of vitamin D$_3$ metabolites is controlled by 24-hydroxylase-mediated inactivation (Jones et al. 1998, Omdahl et al. 2002). The present study demonstrates that 24-hydroxylase expression is induced by both 25OHD$_3$ and 1α,25-(OH)$_2$D$_3$ in stromal, normal epithelial, and cancer epithelial cells in a concentration- and time-dependent manner. Stromal and normal epithelial cells (250 nM 25OHD$_3$ is effective) are more sensitive to 25OHD$_3$ than cancer epithelial cells (≥ 500 nM). Our result also shows that protein synthesis is required in the induction of 24-hydroxylase mRNA by 1α,25-(OH)$_2$D$_3$ in LNCaP cells, which has earlier been reported in other cell lines (Armbrecht et al. 1997, Zierold et al. 2002). A previous study performed on prostate cancer epithelial cells suggested that the growth response was inversely correlated with 24-hydroxylase expression (Miller et al. 1995), which was also observed in our study. 10 nM 1α,25-(OH)$_2$D$_3$ failed to control P32S cell growth, perhaps partially due to the highly induced 24-hydroxylase expression. On the other hand, 25OHD$_3$ induced a much smaller amount of 24-hydroxylase in those cells and the growth was even inhibited by 100 nM 25OHD$_3$. This vitamin D$_3$-resistance phenomenon has been observed in DU145 cells (Ly et al. 1999). We found that in the presence of a 24-hydroxylase inhibitor, VID400, the transcriptional activity of both 25OHD$_3$ and 1α,25-(OH)$_2$D$_3$ in stromal cells is greatly increased, emphasizing the critical role of 24-hydroxylase-mediated inactivation. It has been reported that 24-hydroxylase enzyme

inhibitors enhance the transcriptional activity of 1α,25-$(OH)_2D_3$ in human skin (Kang et al. 1997) as well as the antiproliferative activity of 1α,25-$(OH)_2D_3$ in prostate cancer cells (Ly et al. 1999, Peehl et al. 2002) and ovarian cancer cells (Miettinen et al. 2004). The importance of 24-hydroxylase in vitamin D_3-resistance has been highlighted by a study demonstrating that 24-hydroxylase is a putative oncogene in human breast cancer (Albertson et al. 2000). Amplification of the chromosomal region, where 24-hydroxylase gene is located, has frequently been reported in human ovarian cancer (Tanner et al. 2000), breast cancer (Kallioniemi et al. 1994, Tanner et al. 1995), and prostate cancer (Wolter et al. 2002) and mouse islet carcinomas (Hodgson et al. 2001). The overexpression of 24-hydroxylase due to its amplification may diminish or abolish vitamin D_3-mediated growth control (Albertson et al. 2000). Epidemiological study also suggests that a high level of serum $25OHD_3$ is associated with a higher prostate cancer risk, perhaps due to the development of vitamin D_3-resistance (Tuohimaa et al. 2004). Thus, 24-hydroxylase is a key factor in the successful application of vitamin D_3 metabolites in cancer chemoprevention and therapy.

Taken together, our results indicate that the induction of 24-hydroxylase expression by $25OHD_3$ and 1α,25-$(OH)_2D_3$ seems to be a determinant of vitamin D_3 action in the prostate because the inhibition of 1α-hydroxylase expression by 1α,25-$(OH)_2D_3$ is absent. Although, the 1α,25-$(OH)_2D_3$-induced decrease in 1α-hydroxylase activity has been observed in some extrarenal tissues (Bikle et al. 1986, Delvin and Arabian 1987, Jones et al. 1999, Monkawa et al. 2000, Young et al. 2004), this is, in fact, due to the up-regulation of 24-hydroxylase, which decreases concentrations of both the substrate $25OHD_3$ and the product 1α,25-$(OH)_2D_3$ (Xie et al. 2002). A better understanding of the tissue-specific regulation of vitamin D_3 metabolism and function may help the clinical use of vitamin D_3 metabolites in cancer treatment.

2. INTERACTION BETWEEN ANDROGEN AND VITAMIN D_3

To understand the prostate-specific regulation of vitamin D_3 metabolism and function, we studied the androgen effect on 24-hydroxylase and vitamin D_3-mediated growth control. Androgen plays an important role in the prostate. LNCaP cells are androgen-sensitive and express AR. DHT has been shown to stimulate the growth of LNCaP cells at low concentrations (0.001 to 0.1 nM) and to inhibit it at high concentrations (1 to 100 nM) (Lee et al. 1995, Zhao et al. 1997). Both epidemiological and experimental studies have suggested that androgen signaling is required for the anticancer action of vitamin D_3 (Zhao et al. 1997, Ahonen et al. 2000a, Bao et al. 2004). However, it is not clear how vitamin D_3 and androgen signaling pathways interact. The present study demonstrates that DHT regulates the induction of 24-hydroxylase mRNA levels by $25OHD_3$ or 1α,25-$(OH)_2D_3$ in LNCaP cells in a concentration-dependent manner. DHT at low concentrations (0.01-0.1 nM) slightly increased the levels of the induced 24-hydroxylase mRNA. However, DHT at a physiological concentration of 1 nM caused a 50 %-75 % reduction and 10-100 nM of DHT caused 94 %-99 % suppression. Furthermore, DHT at 10 nM reduced 1α,25-$(OH)_2D_3$ catabolism suggesting that it protects 1α,25-$(OH)_2D_3$ from inactivation and therefore, the local tissue concentration of 1α,25-$(OH)_2D_3$ remains higher. Moreover, our study shows that DHT enhances the antiproliferative activity of $25OHD_3$ and 1α,25-$(OH)_2D_3$. The growth-inhibitory effect of 500 nM $25OHD_3$ and 10 nM 1α,25-$(OH)_2D_3$ is significantly enhanced by 1 nM DHT. Both $25OHD_3$ and 1α,25-$(OH)_2D_3$ at physiological concentrations exhibit strong antiproliferative activity only in the presence of 1 nM DHT. This enhancement by DHT of the

antiproliferative action of vitamin D_3 hormones correlates with the inhibitory effect of DHT on the expression of 24-hydroxylase.

This study may explain the epidemiological result, which shows that $25OHD_3$ has a protective role against prostate cancer only before the andropause, when serum androgen levels are higher (Ahonen et al. 2000a). It also suggests that a combination of androgen and either $25OHD_3$ or $1\alpha,25\text{-}(OH)_2D_3$ at physiological concentrations may be a more beneficial treatment of androgen-dependent prostate cancer than either alone. Because DHT itself has a dose-dependent inhibitory effect on cell growth and may prevent prostate cancer from becoming androgen independent (Feldman and Feldman 2001), the clinical use of DHT in combination with vitamin D_3 metabolites could be more beneficial than the use of other 24-hydroxylase enzyme inhibitors. Although androgen ablation is considered a standard therapy for prostate cancer treatment, the epidemiological studies show no association between high levels of circulating testosterone and increased prostate cancer risk (Chen et al. 2003a, Stattin et al. 2004). In contrast, prostate cancer, especially with more advanced tumors, causes lower levels of both testosterone and DHT (Gustafsson et al. 1996, Hoffman et al. 2000), which may stimulate the growth of prostate cancer cells (Lee et al. 1995, Zhao et al. 1997). The use of androgens in the treatment of prostate cancer has earlier been suggested (Prehn 1999). Based on the present study, the use of either $25OHD_3$ or $1\alpha,25\text{-}(OH)_2D_3$ in combination with androgen at physiological concentrations may reprensent a feasible therapeutic approach for androgen-dependent prostate cancer.

By using AR agonist and antagonist as well as androgen-insensitive cells, we demonstrate that AR is required for the effect of DHT. $1\alpha,25\text{-}(OH)_2D_3$-induced expression of 24-hydroxylase was strongly inhibited by hydroxyflutamide. Hydroxyflutamide functions as an agonist for the mutant AR of LNCaP cells (Veldscholte et al. 1992, Berrevoets et al. 1993). An AR antagonist, Casodex, did not affect $1\alpha,25\text{-}(OH)_2D_3$-induced expression of 24-hydroxylase but partially antagonized the action of DHT because the dose of Casodex (100-fold excess) used can only partially inhibit DHT binding. It has been reported that a 1000-fold excess concentration of Casodex is needed to completely block DHT binding (Zhao et al. 2000). The effect of DHT was not observed in androgen-insensitive human prostate cancer cell lines, DU145 and PC3 whether transfected with the wild-type AR or the mutant AR of LNCaP cells. This indicates that besides AR, there must be other factor(s) involved in the action of androgen.

The mechanism of this action of DHT is not known. Some possible pathways have been excluded. First, DHT does not change VDR mRNA levels. This is consistent with previous reports in other types of cells including prostate cells (Wiese et al. 1992, Li et al. 1999a, Leman and Getzenberg 2003). Second, DHT does not affect the stability of 24-hydroxylase mRNA. Third, no androgen-responsive element has been identified in 24-hydroxylase gene promoter and DHT does not affect the basal level of 24-hydroxylase mRNA. DHT may directly impair the transcriptional function of the liganded VDR, presumably by interfering with coactivator recruitment or by mitogen-activated protein (MAP) kinase activation. There is no earlier report about the interaction of androgen and vitamin D signaling pathways at a specific gene level. Further studies are needed to elucidate the mechanisms underlying the effect of DHT.

3. INTERACTION BETWEEN ALL-TRANS RETINOIC ACID AND VITAMIN D_3

This study demonstrates that ATRA significantly decreases the expression of $25OHD_3$- and $1\alpha,25\text{-}(OH)_2D_3$-induced 24-hydroxylase mRNA levels in primary cultures of human prostate stromal cells P29SN and P32S, but not in either primary culture of human prostate epithelial cells PrEC or cancer epithelial cells LNCaP and PC3. Studies on the transfection of RARβ in PC3 cells and using RAR-specific ligands eliminated the role of RARβ in the action of ATRA. Furthermore, by using an RARα agonist Am80, we found that this action of ATRA is actually mediated by RARα. RARα has been found to be responsible for the action of RA in breast cancer cells (Fitzgerald et al. 1997, Schneider et al. 2000). This inhibitory effect of ATRA in stromal cells may therefore enhance the antiproliferative activity of vitamin D metabolites. Cell growth study showed that the combined treatment of $1\alpha,25\text{-}(OH)_2D_3$ and Am80 at 10 nM strongly inhibits cell growth whereas either alone has no effect. The prostate stroma plays an important role in benign prostatic hyperplasia as well as in carcinogenesis, and aberrant interaction between stroma and epithelium is believed to contribute to carcinoma progression (Tlsty and Hein 2001, Chung et al. 2003, Bhowmick et al. 2004, Mueller and Fusenig 2004). It has been demonstrated that carcinoma-associated fibroblasts do not form tumors but promote carcinogenesis of non-tumorigenic prostate epithelial cells (Grossfeld et al. 1998, Olumi et al. 1999, Cunha et al. 2002).

ATRA, the most potent form of vitamin A, plays an important role in the growth and differentiation of many cells (Altucci and Gronemeyer 2001). ATRA is the main signaling retinoid in the body and exerts its action by binding to RARs, which also act as heterodimers with RXR and bind to the retinoic acid response element (Altucci and Gronemeyer 2001). 9-cis RA binds to both RARs and RXRs (Giguere 1994, Chambon 1996). The role of ATRA in regulating 24-hydroxylase expression is not very clear. An early study showed that RXR-selective ligands stimulate 24-hydroxylase mRNA expression and enzymatic activity in mice (Allegretto et al. 1995). In our experiments, ATRA alone had no effect on 24-hydroxylase expression, suggesting that isomerization of ATRA to 9-cis RA is negligible. There has been no earlier report about the effect of ATRA on prostate cells. Our finding is the first demonstration to show crosstalk between vitamin D and ATRA in prostate stromal cells. 9-cis RA has been reported to reduce $1\alpha,25\text{-}(OH)_2D_3$-induced expression of osteocalcin by diverting RXRs away from VDRs (MacDonald et al. 1993). ATRA and vitamin D have earlier been shown to cooperate to promote differentiation of promyeloid leukemia cells (Brown et al. 1994) and synergistically inhibit the growth of breast cancer cells (Koga and Sutherland 1991, Wang et al. 2000) with different mechanisms.

The present data show that RARα, but not RARβ, mediated ATRA-induced expression of RARβ in prostate stromal cells, which is consistent with other studies performed in cervical cells (Geisen et al. 1997) and breast cancer cells MCF7 (Shang et al. 1999). The higher expression levels of RARβ in stromal cells may result from the more active RARα, which mediates the action of ATRA in reducing 24-hydroxylase expression in stromal cells. Additionally, the present study shows that prostate cancer LNCaP cells express much lower levels of RARs than normal cells, which is consistent with earlier reports showing that the expression of RARs is decreased in tumor progression (Hansen et al. 2000). The loss of responsiveness to ATRA in terms of growth inhibition and RARβ inducibility, as seen in LNCaP cells in our study, has been

reported in colon (Nicke et al. 1999), lung (Zhang et al. 1994) and breast cancers (Liu et al. 1996b). We found that ATRA did not regulate the expression of RARα and RARγ genes in prostate cells, as also in breast cancer cells (Liu et al. 1996b, Shang et al. 1999).

4. NOVEL ENDOCRINE SYSTEM OF 25OHD$_3$

The present study demonstrates that 25OHD$_3$ is an active hormone in the prostate. 25OHD$_3$ (physiological concentrations 20-105 nM) at 100-250 nM in stromal cells and primary epithelial cells or at 500 nM in cancer LNCaP cells can induce 24-hydroxylase expression and inhibit cell growth, whereas 0.1 nM of 1α,25-(OH)$_2$D$_3$ within a physiological concentration range (48-156 pM) is inactive. 25OHD$_3$ at physiological concentration is as effective as 1α,25-(OH)$_2$D$_3$ at pharmacological concentration. By using a specific inhibitor of 1α-hydroxylase, we demonstrate for the first time that 25OHD$_3$ possesses an inherent hormonal activity and that its activation through 1α-hydroxylation is not essential for its biological activity.

1α,25-(OH)$_2$D$_3$ has the greatest binding affinity to VDR (K_d = 90-300 pM for rat VDR) (Walters 1992). 25OHD$_3$ binds to human VDR approximately 50 times less effectively (Bouillon et al. 1995) and to chicken VDR 150-667 times less effectively (Brumbaugh and Haussler 1974, Bouillon et al. 1995). Therefore, 1α,25-(OH)$_2$D$_3$ has been regarded as the vitamin D hormone. However, the serum concentrations of 25OHD$_3$ are approximately 1000-fold greater than those of 1α,25-(OH)$_2$D$_3$, which indicates that the biological activity of the circulating 25OHD$_3$ is significant. Moreover, 24-hydroxylase displays 10-fold greater affinity for 1α,25-(OH)$_2$D$_3$ than 25OHD$_3$ (Chen et al. 1993). It has been suggested that 55 to 90 % of the biological action of vitamin D$_3$ metabolites is mediated by 25OHD$_3$ (Zittermann 2003). 1α,25-(OH)$_2$D$_3$ has been demonstrated *in vitro* to regulate the growth, differentiation, and function of a variety of cells including cancer cells (Mehta and Mehta 2002, Ylikomi et al. 2002, Chen and Holick 2003, Lin and White 2004, Nagpal et al. 2005), but its antiproliferative and differentiation actions are achieved only at hypercalcemic concentrations (Osborn et al. 1995, Gross et al. 1998, Smith et al. 1999).

Based on the present results, we propose a novel vitamin D$_3$ endocrine system (Figure 3), distinct from the classical system involved in calcium homeostasis and mediated by 1α,25-(OH)$_2$D$_3$. The novel vitamin D$_3$ endocrine system is based on the liver hormone, 25OHD$_3$, which regulates cell proliferation and gene expression at physiological concentrations. The synthesis of 1α,25-(OH)$_2$D$_3$ in the kidney is tightly controlled by the hormone itself, PTH, and calcium, through the regulation of 1α-hydroxylase and 24-hydroxylase. Thus, the circulating levels of 1α,25-(OH)$_2$D$_3$ vary within an extremely narrow range not affected by the season (Hine and Roberts 1994, Corder et al. 1995). In contrast, the physiological serum concentration of 25OHD$_3$ fluctuates within a wide range, depending on the season (Hine and Roberts 1994, Corder et al. 1995). The results may also explain those epidemiological studies suggesting that UV radiation and 25OHD$_3$ may protect against prostate cancer (Schwartz and Hulka 1990, Ahonen et al. 2000a, Luscombe et al. 2001) and other cancers (Tangrea et al. 1997, Grant 2002, Bertone-Johnson et al. 2005). However, serum levels of 1α,25-(OH)$_2$D$_3$ are not associated with cancer risk (Tangrea et al. 1997, Bertone-Johnson et al. 2005).

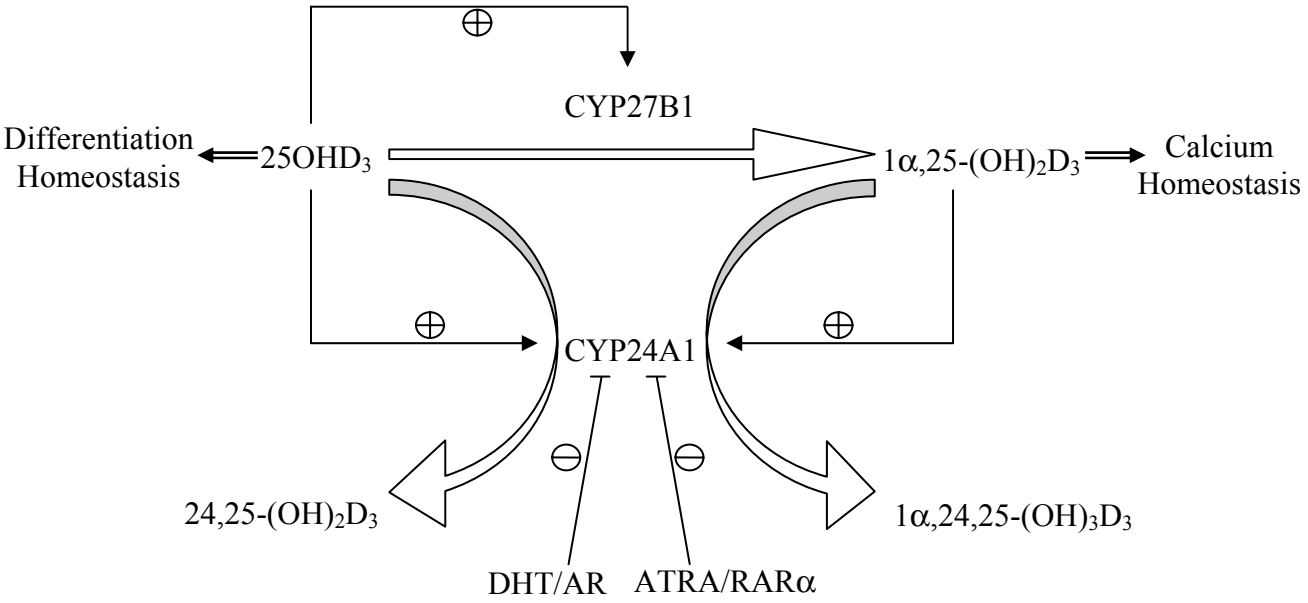


Figure 3. Summary of the results. There are two vitamin D_3 endocrine systems: the classical $1\alpha,25\text{-}(OH)_2D_3$-mediated system is responsible for calcium homeostasis whereas the novel $25OHD_3$-mediated system regulates cell proliferation and differentiation. Both hormones regulate the key enzymes 24-hydroxylase and/or 1α-hydroxylase in the prostate. Androgen and all-trans-retinoic acid enhance the activities of vitamin D hormones by suppressing the expression of 24-hydroxylase in prostate epithelial and stromal cells, respectively.

SUMMARY AND CONCLUSIONS

This study provides evidence for the prostatic expression of two key enzymes involved in vitamin D_3 metabolism, 1α-hydroxylase and 24-hydroxylase. 1α-Hydroxylase is up-regulated by $25OHD_3$ in stromal cells. 24-Hydroxylase is up-regulated by both $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ in epithelial and stromal cells. In the presence of a 24-hydroxylase inhibitor, VID400, the transcriptional activity of both $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ in stromal cells is greatly increased, emphasizing the critical role of 24-hydroxylase-mediated inactivation. Thus, inhibition of 24-hydroxylase is very important aspect in the clinical use of vitamin D_3 metabolites.

To understand the prostate-specific regulation of vitamin D_3 metabolism and function, the androgen effect on 24-hydroxylase and vitamin D_3-mediated growth control was studied. DHT at a physiological concentration enhances the antiproliferative activities of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ by suppressing the expression of 24-hydroxylase in LNCaP cells. This finding provides a feasible therapeutic approach for androgen-dependent prostate cancer.

ATRA significantly decreases the expression of 24-hydroxylase mRNA induced by $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ in primary cultures of human prostate stromal cells P29SN and P32S but not in either primary culture of human prostate epithelial cells PrEC or cancer epithelial cells LNCaP and PC3. With an RARα agonist Am80, RARα was found to mediate the action of ATRA. This inhibitory effect of ATRA in stromal cells may therefore, enhance the antiproliferative activity of vitamin D metabolites. Cell growth study showed that the combined treatment of $1\alpha,25\text{-}(OH)_2D_3$ and Am80 at 10 nM strongly inhibits cell growth whereas either alone has no effect.

The finding that $25OHD_3$ at a physiological concentration possesses an inherent hormonal activity provides a novel view of the vitamin D_3 endocrine system and suggests a potent anticancer therapy. $1\alpha,25\text{-}(OH)_2D_3$ is inactive at its physiological concentrations but pharmacological concentrations are needed for induction of target gene expression and growth inhibition.

Taken together, the present study demonstrates several important aspects regarding the role of vitamin D_3 metabolites and their enzymes in the prevention and treatment of prostate cancer. $25OHD_3$ mediates a distinct vitamin D_3 endocrine system. 24-Hydroxylase is a key factor in the successful application of vitamin D_3 metabolites in cancer chemoprevention and therapy. The crosstalk between vitamin D_3 and DHT or ATRA may provide therapeutic approaches.

ACKNOWLEDGEMENTS

This study was carried out at the Department of Anatomy, Medical School, University of Tampere, Finland.

I am most grateful to my supervisor, Professor Pentti Tuohimaa, M.D., Ph.D., for his patient guidance, never-ending encouragement and generous help. It has been a pleasure to work in his group. His enthusiasm and enormous knowledge of science have been of great value. Without his support, this work would not have been possible.

I would like to thank Docent Anitta Mahonen, Ph.D. and Professor Pirkko Vihko, M.D., Ph.D., the official reviewers of this thesis, for their advice and valuable criticism.

I wish to express my gratitude to Heimo Syvälä, Ph.D., for his kind support and help over the years. I would like to thank Ya-Hua Zhuang, M.D., Ph.D., for his valuable help in life science and computer science. I wish to thank Professor Timo Ylikomi, M.D., Ph.D., for his support and help.

My sincere thanks go to my collaborators and co-authors, Ilkka Laaksi, M.D., Nadja Nazarova, M.Sc., Susanna Miettinen, M.Sc., Merja Bläuer, Ph.D., Riikka Talonpoika, M.D., Hiroyuki Kagechika, Ph.D., Hinrich Gronemeyer, Ph.D., Teuvo L.J. Tammela, M.D., Ph.D., for their contribution to this study.

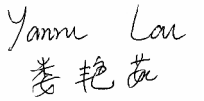
I am grateful to Mrs. Hilkka Mäkinen, Ms. Arja Ahola, Ms. Taina Eskola, for their excellent technical assistance.

My colleagues in the Medical School at University of Tampere are acknowledged for their friendship and help. Special thanks go to Merja Ahonen, Ph.D., Mrs. Tarja Arvela, Olga Golovko, M.Sc., Ms. Mirja Hyppönen, Ulla Järvelin, M.D., Allan V. Kalueff, Ph.D., Ms. Marianne Kuuslahti, Tommi Manninen, M.Sc., Mika Perttu, M.D., Teemu Murtola, M.D., Pasi Pennanen, M.Sc., Sami Purmonen, M.Sc., Shengjun Qiao, M.Sc., Riina Sarkanen, M.Sc., and Annika Vienonen, Ph.D.

Finally, my warmest and loving thanks go to my family and friends for their support and encouragement during these years. I am grateful to my father, Wanzhen, and to my sister, Yanbin, for their endless support and love.

I wish to thank Virginia Mattila, M.A., for revising the language of this thesis. This study was financially supported by the Medical Research Fund of the Tampere University Hospital, the Academy of Finland and the Finnish Cancer Foundation. Permission to republish the original articles was kindly granted by FASEB (I) and John Wiley & Sons, Inc. (II).

Tampere, October 2005



Yan-Ru Lou

REFERENCES

Abate-Shen C and Shen MM (2000): Molecular genetics of prostate cancer. Genes Dev 14**:**2410-2434.

Adams JS, Sharma OP, Gacad MA and Singer FR (1983): Metabolism of 25-hydroxyvitamin D3 by cultured pulmonary alveolar macrophages in sarcoidosis. J Clin Invest 72**:**1856-1860.

Adams JS, Singer FR, Gacad MA, Sharma OP, Hayes MJ, Vouros P and Holick MF (1985): Isolation and structural identification of 1,25-dihydroxyvitamin D3 produced by cultured alveolar macrophages in sarcoidosis. J Clin Endocrinol Metab 60**:**960-966.

Adams JS, Sharma OP, Diz MM and Endres DB (1990): Ketoconazole decreases the serum 1,25-dihydroxyvitamin D and calcium concentration in sarcoidosis-associated hypercalcemia. J Clin Endocrinol Metab 70**:**1090-1095.

Agarwal KS, Mughal MZ, Upadhyay P, Berry JL, Mawer EB and Puliyel JM (2002): The impact of atmospheric pollution on vitamin D status of infants and toddlers in Delhi, India. Arch Dis Child 87**:**111-113.

Ahonen MH, Tenkanen L, Teppo L, Hakama M and Tuohimaa P (2000a): Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland). Cancer Causes Control 11**:**847-852.

Ahonen MH, Zhuang YH, Aine R, Ylikomi T and Tuohimaa P (2000b): Androgen receptor and vitamin D receptor in human ovarian cancer: growth stimulation and inhibition by ligands. Int J Cancer 86**:**40-46.

Akiyoshi-Shibata M, Sakaki T, Ohyama Y, Noshiro M, Okuda K and Yabusaki Y (1994): Further oxidation of hydroxycalcidiol by calcidiol 24-hydroxylase. A study with the mature enzyme expressed in Escherichia coli. Eur J Biochem 224**:**335-343.

Albertson DG, Ylstra B, Segraves R, Collins C, Dairkee SH, Kowbel D, Kuo WL, Gray JW and Pinkel D (2000): Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. Nat Genet 25**:**144-146.

Allegretto EA, Shevde N, Zou A, Howell SR, Boehm MF, Hollis BW and Pike JW (1995): Retinoid X receptor acts as a hormone receptor in vivo to induce a key metabolic enzyme for 1,25-dihydroxyvitamin D3. J Biol Chem 270**:**23906-23909.

Altucci L and Gronemeyer H (2001): The promise of retinoids to fight against cancer. Nat Rev Cancer 1**:**181-193.

Anderson PH, O'Loughlin PD, May BK and Morris HA (2005): Modulation of CYP27B1 and CYP24 mRNA expression in bone is independent of circulating 1,25(OH)2D3 levels. Bone 36**:**654-662.

Araya Z, Tang W and Wikvall K (2003): Hormonal regulation of the human sterol 27-hydroxylase gene CYP27A1. Biochem J 372**:**529-534.

Armbrecht HJ and Boltz MA (1991): Expression of 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 in kidney and intestine. Effect of 1,25-dihydroxyvitamin D and age. FEBS Lett 292**:**17-20.

Armbrecht HJ, Wongsurawat VJ, Hodam TL and Wongsurawat N (1996): Insulin markedly potentiates the capacity of parathyroid hormone to increase expression of 25-hydroxyvitamin D3-24-hydroxylase in rat osteoblastic cells in the presence of 1,25-dihydroxyvitamin D3. FEBS Lett 393**:**77-80.

Armbrecht HJ, Chen ML, Hodam TL and Boltz MA (1997): Induction of 24-hydroxylase cytochrome P450 mRNA by 1,25-dihydroxyvitamin D and phorbol esters in normal rat kidney (NRK-52E) cells. J Endocrinol 153**:**199-205.

Armbrecht HJ, Hodam TL, Boltz MA, Partridge NC, Brown AJ and Kumar VB (1998): Induction of the vitamin D 24-hydroxylase (CYP24) by 1,25- dihydroxyvitamin D3 is regulated by parathyroid hormone in UMR106 osteoblastic cells [see comments]. Endocrinology 139**:**3375-3381.

Axen E, Postlind H and Wikvall K (1995): Effects on CYP27 mRNA expression in rat kidney and liver by 1 alpha, 25-dihydroxyvitamin D3, a suppressor of renal 25-hydroxyvitamin D3 1 alpha-hydroxylase activity. Biochem Biophys Res Commun 215**:**136-141.

Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J and O'Malley BW (1988): Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci U S A 85**:**3294-3298.

Bao BY, Hu YC, Ting HJ and Lee YF (2004): Androgen signaling is required for the vitamin D-mediated growth inhibition in human prostate cancer cells. Oncogene 23**:**3350-3360.

Barletta F, Freedman LP and Christakos S (2002): Enhancement of VDR-mediated transcription by phosphorylation: correlation with increased interaction between the VDR and DRIP205, a subunit of the VDR-interacting protein coactivator complex. Mol Endocrinol 16**:**301-314.

Barletta F, Dhawan P and Christakos S (2004): Integration of hormone signaling in the regulation of human 25(OH)D3 24-hydroxylase transcription. Am J Physiol Endocrinol Metab 286**:**E598-608.

Barreto AM, Schwartz GG, Woodruff R and Cramer SD (2000): 25-Hydroxyvitamin D3, the prohormone of 1,25-dihydroxyvitamin D3, inhibits the proliferation of primary prostatic epithelial cells. Cancer Epidemiol Biomarkers Prev 9**:**265-270.

Beckman MJ, Goff JP, Reinhardt TA, Beitz DC and Horst RL (1994): In vivo regulation of rat intestinal 24-hydroxylase: potential new role of calcitonin. Endocrinology 135**:**1951-1955.

Beckman MJ, Tadikonda P, Werner E, Prahl J, Yamada S and DeLuca HF (1996): Human 25-hydroxyvitamin D3-24-hydroxylase, a multicatalytic enzyme. Biochemistry 35**:**8465-8472.

Bell NH, Greene A, Epstein S, Oexmann MJ, Shaw S and Shary J (1985): Evidence for alteration of the vitamin D-endocrine system in blacks. J Clin Invest 76**:**470-473.

Bemiss CJ, Mahon BD, Henry A, Weaver V and Cantorna MT (2002): Interleukin-2 is one of the targets of 1,25-dihydroxyvitamin D3 in the immune system. Arch Biochem Biophys 402**:**249-254.

Bergh JJ, Xu Y and Farach-Carson MC (2004): Osteoprotegerin expression and secretion are regulated by calcium influx through the L-type voltage-sensitive calcium channel. Endocrinology 145**:**426-436.

Berrevoets CA, Veldscholte J and Mulder E (1993): Effects of antiandrogens on transformation and transcription activation of wild-type and mutated (LNCaP) androgen receptors. J Steroid Biochem Mol Biol 46**:**731-736.

Bertone-Johnson ER, Chen WY, Holick MF, Hollis BW, Colditz GA, Willett WC and Hankinson SE (2005): Plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and risk of breast cancer. Cancer Epidemiol Biomarkers Prev 14**:**1991-1997.

Bhalla AK, Amento EP, Serog B and Glimcher LH (1984): 1,25-Dihydroxyvitamin D3 inhibits antigen-induced T cell activation. J Immunol 133**:**1748-1754.

Bhowmick NA, Neilson EG and Moses HL (2004): Stromal fibroblasts in cancer initiation and progression. Nature 432**:**332-337.

Bia MJ and Insogna K (1991): Treatment of sarcoidosis-associated hypercalcemia with ketoconazole. Am J Kidney Dis 18**:**702-705.

Bianco JJ, Handelsman DJ, Pedersen JS and Risbridger GP (2002): Direct response of the murine prostate gland and seminal vesicles to estradiol. Endocrinology 143**:**4922-4933.

Bikle DD (2004): Vitamin D and skin cancer. J Nutr 134**:**3472S-3478S.

Bikle DD, Nemanic MK, Gee E and Elias P (1986): 1,25-Dihydroxyvitamin D3 production by human keratinocytes. Kinetics and regulation. J Clin Invest 78**:**557-566.

Bikle DD, Pillai S, Gee E and Hincenbergs M (1989): Regulation of 1,25-dihydroxyvitamin D production in human keratinocytes by interferon-gamma. Endocrinology 124**:**655-660.

Bikle DD, Ng D, Oda Y, Hanley K, Feingold K and Xie Z (2002): The vitamin D response element of the involucrin gene mediates its regulation by 1,25-dihydroxyvitamin D3. J Invest Dermatol 119**:**1109-1113.

Bises G, Kallay E, Weiland T, Wrba F, Wenzl E, Bonner E, Kriwanek S, Obrist P and Cross HS (2004): 25-hydroxyvitamin D3-1alpha-hydroxylase expression in normal and malignant human colon. J Histochem Cytochem 52**:**985-989.

Blanco JC, Wang IM, Tsai SY, Tsai MJ, O'Malley BW, Jurutka PW, Haussler MR and Ozato K (1995): Transcription factor TFIIB and the vitamin D receptor cooperatively activate ligand-dependent transcription. Proc Natl Acad Sci U S A 92**:**1535-1539.

Bland R, Walker EA, Hughes SV, Stewart PM and Hewison M (1999): Constitutive expression of 25-hydroxyvitamin D3-1alpha-hydroxylase in a transformed human proximal tubule cell line: evidence for direct regulation of vitamin D metabolism by calcium. Endocrinology 140**:**2027-2034.

Blutt SE, McDonnell TJ, Polek TC and Weigel NL (2000a): Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2. Endocrinology 141**:**10-17.

Blutt SE, Polek TC, Stewart LV, Kattan MW and Weigel NL (2000b): A calcitriol analogue, EB1089, inhibits the growth of LNCaP tumors in nude mice. Cancer Res 60**:**779-782.

Bostwick DG, Burke HB, Djakiew D, Euling S, Ho SM, Landolph J, Morrison H, Sonawane B, Shifflett T, Waters DJ and Timms B (2004): Human prostate cancer risk factors. Cancer 101**:**2371-2490.

Bouillon R, Okamura WH and Norman AW (1995): Structure-function relationships in the vitamin D endocrine system. Endocr Rev 16**:**200-257.

Bouillon R, Carmeliet G, Daci E, Segaert S and Verstuyf A (1998): Vitamin D metabolism and action. Osteoporos Int 8 Suppl 2**:**S13-19.

Bourlon PM, Billaudel B and Faure-Dussert A (1999): Influence of vitamin D3 deficiency and 1,25 dihydroxyvitamin D3 on de novo insulin biosynthesis in the islets of the rat endocrine pancreas. J Endocrinol 160**:**87-95.

Boyan BD, Sylvia VL, McKinney N and Schwartz Z (2003): Membrane actions of vitamin D metabolites 1alpha,25(OH)2D3 and 24R,25(OH)2D3 are retained in growth plate cartilage cells from vitamin D receptor knockout mice. J Cell Biochem 90**:**1207-1223.

Boyle BJ, Zhao XY, Cohen P and Feldman D (2001): Insulin-like growth factor binding protein-3 mediates 1 alpha,25-dihydroxyvitamin d(3) growth inhibition in the LNCaP prostate cancer cell line through p21/WAF1. J Urol 165**:**1319-1324.

Braun MM, Helzlsouer KJ, Hollis BW and Comstock GW (1995): Prostate cancer and prediagnostic levels of serum vitamin D metabolites (Maryland, United States). Cancer Causes Control 6**:**235-239.

Brenza HL, Kimmel-Jehan C, Jehan F, Shinki T, Wakino S, Anazawa H, Suda T and DeLuca HF (1998): Parathyroid hormone activation of the 25-hydroxyvitamin D3-1alpha-hydroxylase gene promoter. Proc Natl Acad Sci U S A 95**:**1387-1391.

Brown TA and DeLuca HF (1990): Phosphorylation of the 1,25-dihydroxyvitamin D3 receptor. A primary event in 1,25-dihydroxyvitamin D3 action. J Biol Chem 265**:**10025-10029.

Brown G, Bunce CM, Rowlands DC and Williams GR (1994): All-trans retinoic acid and 1 alpha,25-dihydroxyvitamin D3 co-operate to promote differentiation of the human promyeloid leukemia cell line HL60 to monocytes. Leukemia 8**:**806-815.

Brown AJ, Dusso A and Slatopolsky E (1999): Vitamin D. Am J Physiol 277**:**F157-175.

Brumbaugh PF and Haussler MR (1974): 1 Alpha,25-dihydroxycholecalciferol receptors in intestine. I. Association of 1 alpha,25-dihydroxycholecalciferol with intestinal mucosa chromatin. J Biol Chem 249**:**1251-1257.

Burmester JK, Wiese RJ, Maeda N and DeLuca HF (1988): Structure and regulation of the rat 1,25-dihydroxyvitamin D3 receptor. Proc Natl Acad Sci U S A 85**:**9499-9502.

Cali JJ and Russell DW (1991): Characterization of human sterol 27-hydroxylase. A mitochondrial cytochrome P-450 that catalyzes multiple oxidation reaction in bile acid biosynthesis. J Biol Chem 266**:**7774-7778.

Campbell MJ, Elstner E, Holden S, Uskokovic M and Koeffler HP (1997): Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D3 analogue involves the induction of p21waf1, p27kip1 and E-cadherin. J Mol Endocrinol 19**:**15-27.

Canning MO, Grotenhuis K, de Wit H, Ruwhof C and Drexhage HA (2001): 1-alpha,25-Dihydroxyvitamin D3 (1,25(OH)(2)D(3)) hampers the maturation of fully active immature dendritic cells from monocytes. Eur J Endocrinol 145**:**351-357.

Cantley LK, Russell J, Lettieri D and Sherwood LM (1985): 1,25-Dihydroxyvitamin D3 suppresses parathyroid hormone secretion from bovine parathyroid cells in tissue culture. Endocrinology 117**:**2114-2119.

Cao X, Ross FP, Zhang L, MacDonald PN, Chappel J and Teitelbaum SL (1993): Cloning of the promoter for the avian integrin beta 3 subunit gene and its regulation by 1,25-dihydroxyvitamin D3. J Biol Chem 268**:**27371-27380.

Carling T, Rastad J, Akerstrom G and Westin G (1998): Vitamin D receptor (VDR) and parathyroid hormone messenger ribonucleic acid levels correspond to polymorphic VDR alleles in human parathyroid tumors. J Clin Endocrinol Metab 83**:**2255-2259.

Chakrabarty S, Wang H, Canaff L, Hendy GN, Appelman H and Varani J (2005): Calcium sensing receptor in human colon carcinoma: interaction with Ca(2+) and 1,25-dihydroxyvitamin D(3). Cancer Res 65**:**493-498.

Chambon P (1996): A decade of molecular biology of retinoic acid receptors. FASEB J 10**:**940-954.

Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, Hennekens CH and Pollak M (1998): Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science 279**:**563-566.

Chen KS and DeLuca HF (1995): Cloning of the human 1 alpha,25-dihydroxyvitamin D-3 24-hydroxylase gene promoter and identification of two vitamin D-responsive elements. Biochim Biophys Acta 1263:1-9.

Chen TL and Feldman D (1985): Retinoic acid modulation of 1,25(OH)2 vitamin D3 receptors and bioresponse in bone cells: species differences between rat and mouse. Biochem Biophys Res Commun 132:74-80.

Chen TC and Holick MF (2003): Vitamin D and prostate cancer prevention and treatment. Trends Endocrinol Metab 14:423-430.

Chen TL, Cone CM, Morey-Holton E and Feldman D (1983): 1 alpha,25-dihydroxyvitamin D3 receptors in cultured rat osteoblast-like cells. Glucocorticoid treatment increases receptor content. J Biol Chem 258:4350-4355.

Chen TL, Hirst MA, Cone CM, Hochberg Z, Tietze HU and Feldman D (1984): 1,25-dihydroxyvitamin D resistance, rickets, and alopecia: analysis of receptors and bioresponse in cultured fibroblasts from patients and parents. J Clin Endocrinol Metab 59:383-388.

Chen KS, Prahl JM and DeLuca HF (1993): Isolation and expression of human 1,25-dihydroxyvitamin D3 24-hydroxylase cDNA. Proc Natl Acad Sci USA 90:4543-4547.

Chen JY, Penco S, Ostrowski J, Balaguer P, Pons M, Starrett JE, Reczek P, Chambon P and Gronemeyer H (1995): RAR-specific agonist/antagonists which dissociate transactivation and AP1 transrepression inhibit anchorage-independent cell proliferation. Embo J 14:1187-1197.

Chen H, Hu B, Allegretto EA and Adams JS (2000a): The vitamin D response element-binding protein. A novel dominant-negative regulator of vitamin D-directed transactivation. J Biol Chem 275:35557-35564.

Chen TC, Schwartz GG, Burnstein KL, Lokeshwar BL and Holick MF (2000b): The in vitro evaluation of 25-hydroxyvitamin D3 and 19-nor-1alpha,25-dihydroxyvitamin D2 as therapeutic agents for prostate cancer. Clin Cancer Res 6:901-908.

Chen CH, Sakai Y and Demay MB (2001): Targeting expression of the human vitamin D receptor to the keratinocytes of vitamin D receptor null mice prevents alopecia. Endocrinology 142:5386-5389.

Chen C, Weiss NS, Stanczyk FZ, Lewis SK, DiTommaso D, Etzioni R, Barnett MJ and Goodman GE (2003a): Endogenous sex hormones and prostate cancer risk: a case-control study nested within the Carotene and Retinol Efficacy Trial. Cancer Epidemiol Biomarkers Prev 12:1410-1416.

Chen H, Hewison M, Hu B and Adams JS (2003b): Heterogeneous nuclear ribonucleoprotein (hnRNP) binding to hormone response elements: a cause of vitamin D resistance. Proc Natl Acad Sci U S A 100:6109-6114.

Chen TC, Wang L, Whitlatch LW, Flanagan JN and Holick MF (2003c): Prostatic 25-hydroxyvitamin D-1alpha-hydroxylase and its implication in prostate cancer. J Cell Biochem 88**:**315-322.

Cheng JB, Motola DL, Mangelsdorf DJ and Russell DW (2003): De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxilase. J Biol Chem 278**:**38084-38093.

Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ and Russell DW (2004): Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. Proc Natl Acad Sci U S A 101**:**7711-7715.

Christakos S, Raval-Pandya M, Wernyj RP and Yang W (1996): Genomic mechanisms involved in the pleiotropic actions of 1,25-dihydroxyvitamin D3. Biochem J 316 (Pt 2)**:**361-371.

Chung LW, Hsieh CL, Law A, Sung SY, Gardner TA, Egawa M, Matsubara S and Zhau HE (2003): New targets for therapy in prostate cancer: modulation of stromal-epithelial interactions. Urology 62**:**44-54.

Cippitelli M and Santoni A (1998): Vitamin D3: a transcriptional modulator of the interferon-gamma gene. Eur J Immunol 28**:**3017-3030.

Clemens TL, Adams JS, Henderson SL and Holick MF (1982): Increased skin pigment reduces the capacity of skin to synthesise vitamin D3. Lancet 1**:**74-76.

Clemens TL, Garrett KP, Zhou XY, Pike JW, Haussler MR and Dempster DW (1988): Immunocytochemical localization of the 1,25-dihydroxyvitamin D3 receptor in target cells. Endocrinology 122**:**1224-1230.

Colston KW and Hansen CM (2002): Mechanisms implicated in the growth regulatory effects of vitamin D in breast cancer. Endocr Relat Cancer 9**:**45-59.

Corder EH, Guess HA, Hulka BS, Friedman GD, Sadler M, Vollmer RT, Lobaugh B, Drezner MK, Vogelman JH and Orentreich N (1993): Vitamin D and prostate cancer: a prediagnostic study with stored sera. Cancer Epidemiol Biomarkers Prev 2**:**467-472.

Corder EH, Friedman GD, Vogelman JH and Orentreich N (1995): Seasonal variation in vitamin D, vitamin D-binding protein, and dehydroepiandrosterone: risk of prostate cancer in black and white men. Cancer Epidemiol Biomarkers Prev 4**:**655-659.

Crescioli C, Maggi M, Luconi M, Vannelli GB, Salerno R, Sinisi AA, Bonaccorsi L, Ferruzzi P, Barni T, Forti G and Serio M (2002): Vitamin D3 analogue inhibits keratinocyte growth factor signaling and induces apoptosis in human prostate cancer cells. Prostate 50**:**15-26.

Crofts LA, Hancock MS, Morrison NA and Eisman JA (1998): Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. Proc Natl Acad Sci U S A 95**:**10529-10534.

Cross HS, Peterlik M, Reddy GS and Schuster I (1997): Vitamin D metabolism in human colon adenocarcinoma-derived Caco-2 cells: expression of 25-hydroxyvitamin D3-1alpha-hydroxylase activity and regulation of side-chain metabolism. J Steroid Biochem Mol Biol 62**:**21-28.

Culine S, Kramar A, Droz JP and Theodore C (1999): Phase II study of all-trans retinoic acid administered intermittently for hormone refractory prostate cancer. J Urol 161**:**173-175.

Cunha GR, Hayward SW and Wang YZ (2002): Role of stroma in carcinogenesis of the prostate. Differentiation 70**:**473-485.

D'Ambrosio D, Cippitelli M, Cocciolo MG, Mazzeo D, Di Lucia P, Lang R, Sinigaglia F and Panina-Bordignon P (1998): Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. J Clin Invest 101**:**252-262.

Dardenne O, Prud'homme J, Arabian A, Glorieux FH and St-Arnaud R (2001): Targeted inactivation of the 25-hydroxyvitamin D(3)-1(alpha)-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets. Endocrinology 142**:**3135-3141.

Dardenne O, Prudhomme J, Hacking SA, Glorieux FH and St-Arnaud R (2003a): Rescue of the pseudo-vitamin D deficiency rickets phenotype of CYP27B1-deficient mice by treatment with 1,25-dihydroxyvitamin D3: biochemical, histomorphometric, and biomechanical analyses. J Bone Miner Res 18**:**637-643.

Dardenne O, Prud'homme J, Hacking SA, Glorieux FH and St-Arnaud R (2003b): Correction of the abnormal mineral ion homeostasis with a high-calcium, high-phosphorus, high-lactose diet rescues the PDDR phenotype of mice deficient for the 25-hydroxyvitamin D-1alpha-hydroxylase (CYP27B1). Bone 32**:**332-340.

Darwish HM and DeLuca HF (1992): Identification of a 1,25-dihydroxyvitamin D3-response element in the 5'-flanking region of the rat calbindin D-9k gene. Proc Natl Acad Sci U S A 89**:**603-607.

Dawson PA and Markovich D (2002): Regulation of the mouse Nas1 promoter by vitamin D and thyroid hormone. Pflugers Arch 444**:**353-359.

DeLuca HF (2004): Overview of general physiologic features and functions of vitamin D. Am J Clin Nutr 80**:**1689S-1696S.

Delvin EE and Arabian A (1987): Kinetics and regulation of 25-hydroxycholecalciferol 1 alpha-hydroxylase from cells isolated from human term decidua. Eur J Biochem 163**:**659-662.

Demay MB, Kiernan MS, DeLuca HF and Kronenberg HM (1992): Sequences in the human parathyroid hormone gene that bind the 1,25- dihydroxyvitamin D3 receptor and mediate transcriptional repression in response to 1,25-dihydroxyvitamin D3. Proc Natl Acad Sci U S A 89**:**8097-8101.

Di Cunto F, Topley G, Calautti E, Hsiao J, Ong L, Seth PK and Dotto GP (1998): Inhibitory function of p21Cip1/WAF1 in differentiation of primary mouse keratinocytes independent of cell cycle control. Science 280**:**1069-1072.

Diaz GD, Paraskeva C, Thomas MG, Binderup L and Hague A (2000a): Apoptosis is induced by the active metabolite of vitamin D3 and its analogue EB1089 in colorectal adenoma and carcinoma cells: possible implications for prevention and therapy. Cancer Res 60**:**2304-2312.

Diaz L, Sanchez I, Avila E, Halhali A, Vilchis F and Larrea F (2000b): Identification of a 25-hydroxyvitamin D3 1alpha-hydroxylase gene transcription product in cultures of human syncytiotrophoblast cells. J Clin Endocrinol Metab 85**:**2543-2549.

Dong X, Craig T, Xing N, Bachman LA, Paya CV, Weih F, McKean DJ, Kumar R and Griffin MD (2003): Direct transcriptional regulation of RelB by 1alpha,25-dihydroxyvitamin D3 and its analogs: physiologic and therapeutic implications for dendritic cell function. J Biol Chem 278**:**49378-49385.

Dormanen MC, Bishop JE, Hammond MW, Okamura WH, Nemere I and Norman AW (1994): Nonnuclear effects of the steroid hormone 1 alpha,25(OH)2-vitamin D3: analogs are able to functionally differentiate between nuclear and membrane receptors. Biochem Biophys Res Commun 201**:**394-401.

Dunlop TW, Vaisanen S, Frank C, Molnar F, Sinkkonen L and Carlberg C (2005): The Human Peroxisome Proliferator-activated Receptor delta Gene is a Primary Target of 1alpha,25-Dihydroxyvitamin D(3) and its Nuclear Receptor. J Mol Biol 349**:**248-260.

Dusso AS, Kamimura S, Gallieni M, Zhong M, Negrea L, Shapiro S and Slatopolsky E (1997): gamma-Interferon-induced resistance to 1,25-(OH)2 D3 in human monocytes and macrophages: a mechanism for the hypercalcemia of various granulomatoses. J Clin Endocrinol Metab 82**:**2222-2232.

Ekman P (2000): The prostate as an endocrine organ: androgens and estrogens. Prostate Suppl 10**:**14-18.

Ellem SJ, Schmitt JF, Pedersen JS, Frydenberg M and Risbridger GP (2004): Local aromatase expression in human prostate is altered in malignancy. J Clin Endocrinol Metab 89**:**2434-2441.

Ensrud KE, Stone K, Cauley JA, White C, Zmuda JM, Nguyen TV, Eisman JA and Cummings SR (1999): Vitamin D receptor gene polymorphisms and the risk of fractures in older women. For the Study of Osteoporotic Fractures Research Group. J Bone Miner Res 14**:**1637-1645.

Esquenet M, Swinnen JV, Heyns W and Verhoeven G (1996): Control of LNCaP proliferation and differentiation: actions and interactions of androgens, 1alpha,25-dihydroxycholecalciferol, all- trans retinoic acid, 9-cis retinoic acid, and phenylacetate. Prostate 28**:**182-194.

Evans RM (1988): The steroid and thyroid hormone receptor superfamily. Science 240**:**889-895.

Falzon M (1996): DNA sequences in the rat parathyroid hormone-related peptide gene responsible for 1,25-dihydroxyvitamin D3-mediated transcriptional repression. Mol Endocrinol 10**:**672-681.

Feldman BJ and Feldman D (2001): The development of androgen-independent prostate cancer. Nat Rev Cancer 1**:**34-45.

Fitzgerald P, Teng M, Chandraratna RA, Heyman RA and Allegretto EA (1997): Retinoic acid receptor alpha expression correlates with retinoid-induced growth inhibition of human breast cancer cells regardless of estrogen receptor status. Cancer Res 57**:**2642-2650.

Fleet JC (2004): Rapid, membrane-initiated actions of 1,25 dihydroxyvitamin D: what are they and what do they mean? J Nutr 134**:**3215-3218.

Fong CJ, Sutkowski DM, Braun EJ, Bauer KD, Sherwood ER, Lee C and Kozlowski JM (1993): Effect of retinoic acid on the proliferation and secretory activity of androgen-responsive prostatic carcinoma cells. J Urol 149**:**1190-1194.

Freedman DM, Dosemeci M and McGlynn K (2002): Sunlight and mortality from breast, ovarian, colon, prostate, and non-melanoma skin cancer: a composite death certificate based case-control study. Occup Environ Med 59**:**257-262.

Fritsche J, Mondal K, Ehrnsperger A, Andreesen R and Kreutz M (2003): Regulation of 25-hydroxyvitamin D3-1 alpha-hydroxylase and production of 1 alpha,25-dihydroxyvitamin D3 by human dendritic cells. Blood 102**:**3314-3316.

Fu GK, Lin D, Zhang MY, Bikle DD, Shackleton CH, Miller WL and Portale AA (1997): Cloning of human 25-hydroxyvitamin D-1 alpha-hydroxylase and mutations causing vitamin D-dependent rickets type 1. Mol Endocrinol 11**:**1961-1970.

Gacad MA and Adams JS (1998): Proteins in the heat shock-70 family specifically bind 25-hydroxyvitamin D3 and 17beta-estradiol. J Clin Endocrinol Metab 83**:**1264-1267.

Gacad MA, Chen H, Arbelle JE, LeBon T and Adams JS (1997): Functional characterization and purification of an intracellular vitamin D-binding protein in vitamin D-resistant new world primate cells. Amino acid sequence homology with proteins in the hsp-70 family. J Biol Chem 272**:**8433-8440.

Gann PH, Ma J, Hennekens CH, Hollis BW, Haddad JG and Stampfer MJ (1996): Circulating vitamin D metabolites in relation to subsequent development of prostate cancer. Cancer Epidemiol Biomarkers Prev 5**:**121-126.

Gao XH, Dwivedi PP, Omdahl JL, Morris HA and May BK (2004): Calcitonin stimulates expression of the rat 25-hydroxyvitamin D3-24-hydroxylase (CYP24) promoter in HEK-293 cells expressing calcitonin receptor: identification of signaling pathways. J Mol Endocrinol 32**:**87-98.

Garnero P, Borel O, Sornay-Rendu E, Arlot ME and Delmas PD (1996): Vitamin D receptor gene polymorphisms are not related to bone turnover, rate of bone loss, and bone mass in postmenopausal women: the OFELY Study. J Bone Miner Res 11**:**827-834.

Gascon-Barre M, Demers C, Ghrab O, Theodoropoulos C, Lapointe R, Jones G, Valiquette L and Menard D (2001): Expression of CYP27A, a gene encoding a vitamin D-25 hydroxylase in human liver and kidney. Clin Endocrinol (Oxf) 54**:**107-115.

Geisen C, Denk C, Gremm B, Baust C, Karger A, Bollag W and Schwarz E (1997): High-level expression of the retinoic acid receptor beta gene in normal cells of the uterine cervix is regulated by the retinoic acid receptor alpha and is abnormally down-regulated in cervical carcinoma cells. Cancer Res 57**:**1460-1467.

Gensure RC, Antrobus SD, Fox J, Okwueze M, Talton SY and Walters MR (1998): Homologous up-regulation of vitamin D receptors is tissue specific in the rat. J Bone Miner Res 13**:**454-463.

Germain P, Kammerer S, Perez E, Peluso-Iltis C, Tortolani D, Zusi FC, Starrett J, Lapointe P, Daris JP, Marinier A, de Lera AR, Rochel N and Gronemeyer H (2004): Rational design of RAR-selective ligands revealed by RARbeta crystal stucture. EMBO Rep 5**:**877-882.

Getzenberg RH, Light BW, Lapco PE, Konety BR, Nangia AK, Acierno JS, Dhir R, Shurin Z, Day RS, Trump DL and Johnson CS (1997): Vitamin D inhibition of prostate adenocarcinoma growth and metastasis in the Dunning rat prostate model system. Urology 50**:**999-1006.

Giguere V (1994): Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. Endocr Rev 15**:**61-79.

Gill RK and Christakos S (1993): Identification of sequence elements in mouse calbindin-D28k gene that confer 1,25-dihydroxyvitamin D3- and butyrate-inducible responses. Proc Natl Acad Sci U S A 90**:**2984-2988.

Giuliani DL and Boland RL (1984): Effects of vitamin D3 metabolites on calcium fluxes in intact chicken skeletal muscle and myoblasts cultured in vitro. Calcif Tissue Int 36**:**200-205.

Gniadecki R (1997): Effects of 1,25-dihydroxyvitamin D3 and its 20-epi analogues (MC 1288, MC 1301, KH 1060), on clonal keratinocyte growth: evidence for differentiation of keratinocyte stem cells and analysis of the modulatory effects of cytokines. Br J Pharmacol 120**:**1119-1127.

Goossens K, Esquenet M, Swinnen JV, Manin M, Rombauts W and Verhoeven G (1999): Androgens decrease and retinoids increase the expression of insulin-like growth factor-binding protein-3 in LNcaP prostatic adenocarcinoma cells. Mol Cell Endocrinol 155**:**9-18.

Grant WB (2002): An estimate of premature cancer mortality in the U.S. due to inadequate doses of solar ultraviolet-B radiation. Cancer 94**:**1867-1875.

Griffin MD, Lutz W, Phan VA, Bachman LA, McKean DJ and Kumar R (2001): Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent

pathway that promotes a persistent state of immaturity in vitro and in vivo. Proc Natl Acad Sci U S A 98:6800-6805.

Gronberg H (2003): Prostate cancer epidemiology. Lancet 361:859-864.

Gross C, Stamey T, Hancock S and Feldman D (1998): Treatment of early recurrent prostate cancer with 1,25-dihydroxyvitamin D3 (calcitriol). J Urol 159:2035-2039; discussion 2039-2040.

Grossfeld GD, Hayward SW, Tlsty TD and Cunha GR (1998): The role of stroma in prostatic carcinogenesis. Endocr Relat Cancer 5:253-270.

Guo YD, Strugnell S, Back DW and Jones G (1993): Transfected human liver cytochrome P-450 hydroxylates vitamin D analogs at different side-chain positions. Proc Natl Acad Sci U S A 90:8668-8672.

Guo B, Aslam F, van Wijnen AJ, Roberts SG, Frenkel B, Green MR, DeLuca H, Lian JB, Stein GS and Stein JL (1997): YY1 regulates vitamin D receptor/retinoid X receptor mediated transactivation of the vitamin D responsive osteocalcin gene. Proc Natl Acad Sci U S A 94:121-126.

Gupta RP, Hollis BW, Patel SB, Patrick KS and Bell NH (2004): CYP3A4 is a human microsomal vitamin D 25-hydroxylase. J Bone Miner Res 19:680-688.

Gupta RP, He YA, Patrick KS, Halpert JR and Bell NH (2005): CYP3A4 Is a Vitamin D-24- and 25-Hydroxylase: Analysis of Structure Function by Site-Directed Mutagenesis. J Clin Endocrinol Metab 90:1210-1219.

Gurlek A, Pittelkow MR and Kumar R (2002): Modulation of growth factor/cytokine synthesis and signaling by 1alpha,25-dihydroxyvitamin D(3): implications in cell growth and differentiation. Endocr Rev 23:763-786.

Gustafsson O, Norming U, Gustafsson S, Eneroth P, Astrom G and Nyman CR (1996): Dihydrotestosterone and testosterone levels in men screened for prostate cancer: a study of a randomized population. Br J Urol 77:433-440.

Guzey M, Kitada S and Reed JC (2002): Apoptosis induction by 1alpha,25-dihydroxyvitamin D3 in prostate cancer. Mol Cancer Ther 1:667-677.

Hakim I and Bar-Shavit Z (2003): Modulation of TNF-alpha expression in bone marrow macrophages: involvement of vitamin D response element. J Cell Biochem 88:986-998.

Hammond LA, Van Krinks CH, Durham J, Tomkins SE, Burnett RD, Jones EL, Chandraratna RA and Brown G (2001): Antagonists of retinoic acid receptors (RARs) are potent growth inhibitors of prostate carcinoma cells. Br J Cancer 85:453-462.

Hanchette CL and Schwartz GG (1992): Geographic patterns of prostate cancer mortality. Evidence for a protective effect of ultraviolet radiation. Cancer 70:2861-2869.

Hansen LA, Sigman CC, Andreola F, Ross SA, Kelloff GJ and De Luca LM (2000): Retinoids in chemoprevention and differentiation therapy. Carcinogenesis 21**:**1271-1279.

Harkonen PL and Makela SI (2004): Role of estrogens in development of prostate cancer. J Steroid Biochem Mol Biol 92**:**297-305.

Harman SM, Metter EJ, Blackman MR, Landis PK and Carter HB (2000): Serum levels of insulin-like growth factor I (IGF-I), IGF-II, IGF-binding protein-3, and prostate-specific antigen as predictors of clinical prostate cancer. J Clin Endocrinol Metab 85**:**4258-4265.

Harrison JR, Petersen DN, Lichtler AC, Mador AT, Rowe DW and Kream BE (1989): 1,25-Dihydroxyvitamin D3 inhibits transcription of type I collagen genes in the rat osteosarcoma cell line ROS 17/2.8. Endocrinology 125**:**327-333.

Haussler MR, Myrtle JF and Norman AW (1968): The association of a metabolite of vitamin D3 with intestinal mucosa chromatin in vivo. J Biol Chem 243**:**4055-4064.

Hedlund TE, Moffatt KA and Miller GJ (1996a): Vitamin D receptor expression is required for growth modulation by 1 alpha,25-dihydroxyvitamin D3 in the human prostatic carcinoma cell line ALVA-31. J Steroid Biochem Mol Biol 58**:**277-288.

Hedlund TE, Moffatt KA and Miller GJ (1996b): Stable expression of the nuclear vitamin D receptor in the human prostatic carcinoma cell line JCA-1: evidence that the antiproliferative effects of 1 alpha, 25-dihydroxyvitamin D3 are mediated exclusively through the genomic signaling pathway. Endocrinology 137**:**1554-1561.

Henry HL and Norman AW (1978): Vitamin D: two dihydroxylated metabolites are required for normal chicken egg hatchability. Science 201**:**835-837.

Henttu P and Vihko P (1992): Steroids inversely affect the biosynthesis and secretion of human prostatic acid phosphatase and prostate-specific antigen in the LNCaP cell line. J Steroid Biochem Mol Biol 41**:**349-360.

Henttu P, Liao SS and Vihko P (1992): Androgens up-regulate the human prostate-specific antigen messenger ribonucleic acid (mRNA), but down-regulate the prostatic acid phosphatase mRNA in the LNCaP cell line. Endocrinology 130**:**766-772.

Hershberger PA, Modzelewski RA, Shurin ZR, Rueger RM, Trump DL and Johnson CS (1999): 1,25-Dihydroxycholecalciferol (1,25-D3) inhibits the growth of squamous cell carcinoma and down-modulates p21(Waf1/Cip1) in vitro and in vivo. Cancer Res 59**:**2644-2649.

Hewison M, Freeman L, Hughes SV, Evans KN, Bland R, Eliopoulos AG, Kilby MD, Moss PA and Chakraverty R (2003): Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells. J Immunol 170**:**5382-5390.

Hewison M, Zehnder D, Chakraverty R and Adams JS (2004): Vitamin D and barrier function: a novel role for extra-renal 1 alpha-hydroxylase. Mol Cell Endocrinol 215**:**31-38.

Hine TJ and Roberts NB (1994): Seasonal variation in serum 25-hydroxy vitamin D3 does not affect 1,25-dihydroxy vitamin D. Ann Clin Biochem 31**:**31-34.

Hiramatsu M, Maehara I, Ozaki M, Harada N, Orikasa S and Sasano H (1997): Aromatase in hyperplasia and carcinoma of the human prostate. Prostate 31**:**118-124.

Hodgson G, Hager JH, Volik S, Hariono S, Wernick M, Moore D, Nowak N, Albertson DG, Pinkel D, Collins C, Hanahan D and Gray JW (2001): Genome scanning with array CGH delineates regional alterations in mouse islet carcinomas. Nat Genet 29**:**459-464.

Hoenderop JG, Muller D, Van Der Kemp AW, Hartog A, Suzuki M, Ishibashi K, Imai M, Sweep F, Willems PH, Van Os CH and Bindels RJ (2001): Calcitriol controls the epithelial calcium channel in kidney. J Am Soc Nephrol 12**:**1342-1349.

Hoenderop JG, Nilius B and Bindels RJ (2005): Calcium absorption across epithelia. Physiol Rev 85**:**373-422.

Hofbauer LC and Heufelder AE (2001): Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. J Mol Med 79**:**243-253.

Hoffman MA, DeWolf WC and Morgentaler A (2000): Is low serum free testosterone a marker for high grade prostate cancer? J Urol 163**:**824-827.

Houle B, Rochette-Egly C and Bradley WE (1993): Tumor-suppressive effect of the retinoic acid receptor beta in human epidermoid lung cancer cells. Proc Natl Acad Sci U S A 90**:**985-989.

Houston LA, Grant SF, Reid DM and Ralston SH (1996): Vitamin D receptor polymorphism, bone mineral density, and osteoporotic vertebral fracture: studies in a UK population. Bone 18**:**249-252.

Howard A, Legon S, Spurr NK and Walters JR (1992): Molecular cloning and chromosomal assignment of human calbindin-D9k. Biochem Biophys Res Commun 185**:**663-669.

Hsieh T and Wu JM (1997): Induction of apoptosis and altered nuclear/cytoplasmic distribution of the androgen receptor and prostate-specific antigen by 1alpha,25-dihydroxyvitamin D3 in androgen-responsive LNCaP cells. Biochem Biophys Res Commun 235**:**539-544.

Hsieh TY, Ng CY, Mallouh C, Tazaki H and Wu JM (1996): Regulation of growth, PSA/PAP and androgen receptor expression by 1 alpha,25-dihydroxyvitamin D3 in the androgen-dependent LNCaP cells. Biochem Biophys Res Commun 223**:**141-146.

Hsieh JC, Shimizu Y, Minoshima S, Shimizu N, Haussler CA, Jurutka PW and Haussler MR (1998): Novel nuclear localization signal between the two DNA-binding zinc fingers in the human vitamin D receptor. J Cell Biochem 70**:**94-109.

Hsieh JC, Sisk JM, Jurutka PW, Haussler CA, Slater SA, Haussler MR and Thompson CC (2003): Physical and functional interaction between the vitamin D receptor and hairless corepressor, two proteins required for hair cycling. J Biol Chem 278**:**38665-38674.

Hsieh JC, Dang HT, Galligan MA, Whitfield GK, Haussler CA, Jurutka PW and Haussler MR (2004): Phosphorylation of human vitamin D receptor serine-182 by PKA suppresses 1,25(OH)2D3-dependent transactivation. Biochem Biophys Res Commun 324**:**801-809.

Hsing AW and Comstock GW (1993): Serological precursors of cancer: serum hormones and risk of subsequent prostate cancer. Cancer Epidemiol Biomarkers Prev 2**:**27-32.

Hsu JY, Feldman D, McNeal JE and Peehl DM (2001): Reduced 1alpha-hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D3-induced growth inhibition. Cancer Res 61**:**2852-2856.

Huhtakangas JA, Olivera CJ, Bishop JE, Zanello LP and Norman AW (2004): The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1 alpha,25(OH)2-vitamin D3 in vivo and in vitro. Mol Endocrinol 18**:**2660-2671.

Huldschinsky K (1919): Curing rickets by artificial UV-radiation. Deut Med Wochenschr 45**:**712-713.

Huss WJ, Lai L, Barrios RJ, Hirschi KK and Greenberg NM (2004): Retinoic acid slows progression and promotes apoptosis of spontaneous prostate cancer. Prostate 61**:**142-152.

Hustmyer FG, Peacock M, Hui S, Johnston CC and Christian J (1994): Bone mineral density in relation to polymorphism at the vitamin D receptor gene locus. J Clin Invest 94**:**2130-2134.

Issa LL, Leong GM and Eisman JA (1998): Molecular mechanism of vitamin D receptor action. Inflamm Res 47**:**451-475.

James SY, Mercer E, Brady M, Binderup L and Colston KW (1998): EB1089, a synthetic analogue of vitamin D, induces apoptosis in breast cancer cells in vivo and in vitro. Br J Pharmacol 125**:**953-962.

Jarred RA, Cancilla B, Prins GS, Thayer KA, Cunha GR and Risbridger GP (2000): Evidence that estrogens directly alter androgen-regulated prostate development. Endocrinology 141**:**3471-3477.

Jensen SS, Madsen MW, Lukas J, Binderup L and Bartek J (2001): Inhibitory effects of 1alpha,25-dihydroxyvitamin D(3) on the G(1)-S phase-controlling machinery. Mol Endocrinol 15**:**1370-1380.

Jeronimo C, Henrique R, Hoque MO, Ribeiro FR, Oliveira J, Fonseca D, Teixeira MR, Lopes C and Sidransky D (2004): Quantitative RARbeta2 hypermethylation: a promising prostate cancer marker. Clin Cancer Res 10**:**4010-4014.

Jeung EB, Krisinger J, Dann JL and Leung PC (1992): Molecular cloning of the full-length cDNA encoding the human calbindin-D9k. FEBS Lett 307**:**224-228.

Johnson JA, Beckman MJ, Pansini-Porta A, Christakos S, Bruns ME, Beitz DC, Horst RL and Reinhardt TA (1995): Age and gender effects on 1,25-dihydroxyvitamin D3-regulated gene expression. Exp Gerontol 30**:**631-643.

Jones HE, Eaton CL, Barrow D, Dutkowski C and Griffiths K (1997): Response of cell growth and retinoic acid receptor expression to retinoic acid in neoplastic and non-neoplastic prostate cell lines. Prostate 30**:**174-182.

Jones G, Strugnell SA and DeLuca HF (1998): Current understanding of the molecular actions of vitamin D. Physiol Rev 78**:**1193-1231.

Jones G, Ramshaw H, Zhang A, Cook R, Byford V, White J and Petkovich M (1999): Expression and activity of vitamin D-metabolizing cytochrome P450s (CYP1alpha and CYP24) in human nonsmall cell lung carcinomas. Endocrinology 140**:**3303-3310.

Kallioniemi A, Kallioniemi OP, Piper J, Tanner M, Stokke T, Chen L, Smith HS, Pinkel D, Gray JW and Waldman FM (1994): Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. Proc Natl Acad Sci USA 91**:**2156-2160.

Kamei Y, Kawada T, Fukuwatari T, Ono T, Kato S and Sugimoto E (1995): Cloning and sequencing of the gene encoding the mouse vitamin D receptor. Gene 152**:**281-282.

Kamimura S, Gallieni M, Zhong M, Beron W, Slatopolsky E and Dusso A (1995): Microtubules mediate cellular 25-hydroxyvitamin D3 trafficking and the genomic response to 1,25-dihydroxyvitamin D3 in normal human monocytes. J Biol Chem 270**:**22160-22166.

Kang S, Li XY, Duell EA and Voorhees JJ (1997): The retinoid X receptor agonist 9-cis-retinoic acid and the 24-hydroxylase inhibitor ketoconazole increase activity of 1,25-dihydroxyvitamin D3 in human skin in vivo. J Invest Dermatol 108**:**513-518.

Keedwell RG, Zhao Y, Hammond LA, Wen K, Qin S, Atangan LI, Shurland DL, Wallace DM, Bird R, Reitmair A, Chandraratna RA and Brown G (2004): An antagonist of retinoic acid receptors more effectively inhibits growth of human prostate cancer cells than normal prostate epithelium. Br J Cancer 91**:**580-588.

Kelly WK, Osman I, Reuter VE, Curley T, Heston WD, Nanus DM and Scher HI (2000): The development of biologic end points in patients treated with differentiation agents: an experience of retinoids in prostate cancer. Clin Cancer Res 6**:**838-846.

Kerner SA, Scott RA and Pike JW (1989): Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D3. Proc Natl Acad Sci U S A 86**:**4455-4459.

Khorasanizadeh S and Rastinejad F (2001): Nuclear-receptor interactions on DNA-response elements. Trends Biochem Sci 26**:**384-390.

Kikugawa T, Tanji N, Miyazaki T and Yokoyama M (2000): Immunohistochemical study of the receptors for retinoic acid in prostatic adenocarcinoma. Anticancer Res 20**:**3897-3902.

Kitazawa R and Kitazawa S (2002): Vitamin D(3) augments osteoclastogenesis via vitamin D-responsive element of mouse RANKL gene promoter. Biochem Biophys Res Commun 290**:**650-655.

Kitazawa S, Kajimoto K, Kondo T and Kitazawa R (2003): Vitamin D3 supports osteoclastogenesis via functional vitamin D response element of human RANKL gene promoter. J Cell Biochem 89**:**771-777.

Koga M and Sutherland RL (1991): Retinoic acid acts synergistically with 1,25-dihydroxyvitamin D3 or antioestrogen to inhibit T-47D human breast cancer cell proliferation. J Steroid Biochem Mol Biol 39**:**455-460.

Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, Trapman J, Cleutjens K, Noordzij A, Visakorpi T and Kallioniemi OP (1997): Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 57**:**314-319.

Koli K and Keski-Oja J (1995): 1,25-Dihydroxyvitamin D3 enhances the expression of transforming growth factor beta 1 and its latent form binding protein in cultured breast carcinoma cells. Cancer Res 55**:**1540-1546.

Kong XF, Zhu XH, Pei YL, Jackson DM and Holick MF (1999): Molecular cloning, characterization, and promoter analysis of the human 25-hydroxyvitamin D3-1alpha-hydroxylase gene. Proc Natl Acad Sci U S A 96**:**6988-6993.

Kreutz M, Andreesen R, Krause SW, Szabo A, Ritz E and Reichel H (1993): 1,25-dihydroxyvitamin D3 production and vitamin D3 receptor expression are developmentally regulated during differentiation of human monocytes into macrophages. Blood 82**:**1300-1307.

Krishnan AV and Feldman D (1991): Activation of protein kinase-C inhibits vitamin D receptor gene expression. Mol Endocrinol 5**:**605-612.

Krishnan AV and Feldman D (1992): Cyclic adenosine 3',5'-monophosphate up-regulates 1,25-dihydroxyvitamin D3 receptor gene expression and enhances hormone action. Mol Endocrinol 6**:**198-206.

Krishnan AV, Cramer SD, Bringhurst FR and Feldman D (1995): Regulation of 1,25-dihydroxyvitamin D3 receptors by parathyroid hormone in osteoblastic cells: role of second messenger pathways. Endocrinology 136**:**705-712.

Kueng W, Silber E and Eppenberger U (1989): Quantification of cells cultured on 96-well plates. Anal Biochem 182**:**16-19.

Kutuzova GD and Deluca HF (2004): Gene expression profiles in rat intestine identify pathways for 1,25-dihydroxyvitamin D(3) stimulated calcium absorption and clarify its immunomodulatory properties. Arch Biochem Biophys 432**:**152-166.

Kyeyune-Nyombi E, Lau KH, Baylink DJ and Strong DD (1991): 1,25-Dihydroxyvitamin D3 stimulates both alkaline phosphatase gene transcription and mRNA stability in human bone cells. Arch Biochem Biophys 291**:**316-325.

Lamprecht SA and Lipkin M (2003): Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. Nat Rev Cancer 3**:**601-614.

Lee C, Sutkowski DM, Sensibar JA, Zelner D, Kim I, Amsel I, Shaw N, Prins GS and Kozlowski JM (1995): Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells, LNCaP, by dihydrotestosterone. Endocrinology 136**:**796-803.

Leheste JR, Melsen F, Wellner M, Jansen P, Schlichting U, Renner-Muller I, Andreassen TT, Wolf E, Bachmann S, Nykjaer A and Willnow TE (2003): Hypocalcemia and osteopathy in mice with kidney-specific megalin gene defect. Faseb J 17**:**247-249.

Leman ES and Getzenberg RH (2003): Effects of 1,25-dihydroxyvitamin D3 on the distribution of androgen and vitamin D receptors in human prostate neonatal epithelial cells. J Cell Biochem 88**:**609-622.

Lemay J, Demers C, Hendy GN, Delvin EE and Gascon-Barre M (1995): Expression of the 1,25-dihydroxyvitamin D3-24-hydroxylase gene in rat intestine: response to calcium, vitamin D3 and calcitriol administration in vivo. J Bone Miner Res 10**:**1148-1157.

Lemire J (1997): The role of vitamin D_3 in immunosuppression: lessons from autoimmunity and transplantation. In: Vitamin D*,* pp. 1167-1181. Eds. D Feldman, FH Glorieux and JW Pike, Academic Press, San Diego.

Lemire J (2000): 1,25-Dihydroxyvitamin D3--a hormone with immunomodulatory properties. Z Rheumatol 59 Suppl 1**:**24-27.

Li JJ and Sodek J (1993): Cloning and characterization of the rat bone sialoprotein gene promoter. Biochem J 289**:**625-629.

Li H, Leo C, Schroen DJ and Chen JD (1997a): Characterization of receptor interaction and transcriptional repression by the corepressor SMRT. Mol Endocrinol 11**:**2025-2037.

Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R and Demay MB (1997b): Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. Proc Natl Acad Sci U S A 94**:**9831-9835.

Li YC, Amling M, Pirro AE, Priemel M, Meuse J, Baron R, Delling G and Demay MB (1998): Normalization of mineral ion homeostasis by dietary means prevents hyperparathyroidism, rickets, and osteomalacia, but not alopecia in vitamin D receptor-ablated mice. Endocrinology 139**:**4391-4396.

Li XY, Boudjelal M, Xiao JH, Peng ZH, Asuru A, Kang S, Fisher GJ and Voorhees JJ (1999a): 1,25-Dihydroxyvitamin D3 increases nuclear vitamin D3 receptors by blocking ubiquitin/proteasome-mediated degradation in human skin. Mol Endocrinol 13**:**1686-1694.

Li Y, Hashimoto Y, Agadir A, Kagechika H and Zhang X (1999b): Identification of a novel class of retinoic acid receptor beta-selective retinoid antagonists and their inhibitory effects on AP-1 activity and retinoic acid-induced apoptosis in human breast cancer cells. J Biol Chem 274**:**15360-15366.

Li P, Li C, Zhao X, Zhang X, Nicosia SV and Bai W (2004): p27(Kip1) stabilization and G(1) arrest by 1,25-dihydroxyvitamin D(3) in ovarian cancer cells mediated through down-regulation of cyclin E/cyclin-dependent kinase 2 and Skp1-Cullin-F-box protein/Skp2 ubiquitin ligase. J Biol Chem 279**:**25260-25267.

Liel Y, Kraus S, Levy J and Shany S (1992): Evidence that estrogens modulate activity and increase the number of 1,25-dihydroxyvitamin D receptors in osteoblast-like cells (ROS 17/2.8). Endocrinology 130**:**2597-2601.

Lim SK, Park YS, Park JM, Song YD, Lee EJ, Kim KR, Lee HC and Huh KB (1995): Lack of association between vitamin D receptor genotypes and osteoporosis in Koreans. J Clin Endocrinol Metab 80**:**3677-3681.

Lin R and White JH (2004): The pleiotropic actions of vitamin D. Bioessays 26**:**21-28.

Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL and Visakorpi T (2001): Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res 61**:**3550-3555.

Linja MJ, Savinainen KJ, Tammela TL, Isola JJ and Visakorpi T (2003): Expression of ERalpha and ERbeta in prostate cancer. Prostate 55**:**180-186.

Liu M, Lee MH, Cohen M, Bommakanti M and Freedman LP (1996a): Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev 10**:**142-153.

Liu Y, Lee MO, Wang HG, Li Y, Hashimoto Y, Klaus M, Reed JC and Zhang X (1996b): Retinoic acid receptor beta mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. Mol Cell Biol 16**:**1138-1149.

Lokeshwar BL, Schwartz GG, Selzer MG, Burnstein KL, Zhuang SH, Block NL and Binderup L (1999): Inhibition of prostate cancer metastasis in vivo: a comparison of 1,25- dihydroxyvitamin D (calcitriol) and EB1089. Cancer Epidemiol Biomarkers Prev 8**:**241-248.

Lotan Y, Xu XC, Shalev M, Lotan R, Williams R, Wheeler TM, Thompson TC and Kadmon D (2000): Differential expression of nuclear retinoid receptors in normal and malignant prostates. J Clin Oncol 18**:**116-121.

Luscombe CJ, Fryer AA, French ME, Liu S, Saxby MF, Jones PW and Strange RC (2001): Exposure to ultraviolet radiation: association with susceptibility and age at presentation with prostate cancer. Lancet 358**:**641-642.

Ly LH, Zhao XY, Holloway L and Feldman D (1999): Liarozole acts synergistically with 1alpha,25-dihydroxyvitamin D3 to inhibit growth of DU 145 human prostate cancer cells by blocking 24- hydroxylase activity. Endocrinology 140**:**2071-2076.

Lyakh LA, Sanford M, Chekol S, Young HA and Roberts AB (2005): TGF-beta and vitamin D3 utilize distinct pathways to suppress IL-12 production and modulate rapid differentiation of human monocytes into CD83+ dendritic cells. J Immunol 174**:**2061-2070.

Maas RM, Reus K, Diesel B, Steudel WI, Feiden W, Fischer U and Meese E (2001): Amplification and expression of splice variants of the gene encoding the P450 cytochrome 25-hydroxyvitamin D(3) 1,alpha-hydroxylase (CYP 27B1) in human malignant glioma. Clin Cancer Res 7**:**868-875.

MacDonald PN, Dowd DR, Nakajima S, Galligan MA, Reeder MC, Haussler CA, Ozato K and Haussler MR (1993): Retinoid X receptors stimulate and 9-cis retinoic acid inhibits 1,25-dihydroxyvitamin D3-activated expression of the rat osteocalcin gene. Mol Cell Biol 13**:**5907-5917.

Machtens S, Schultheiss D, Kuczyk M, Truss MC and Jonas U (2000): The history of endocrine therapy of benign and malignant diseases of the prostate. World J Urol 18**:**222-226.

MacLaughlin J and Holick MF (1985): Aging decreases the capacity of human skin to produce vitamin D3. J Clin Invest 76**:**1536-1538.

Maestro B, Davila N, Carranza MC and Calle C (2003): Identification of a Vitamin D response element in the human insulin receptor gene promoter. J Steroid Biochem Mol Biol 84**:**223-230.

Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR and Mangelsdorf DJ (2002): Vitamin D receptor as an intestinal bile acid sensor. Science 296**:**1313-1316.

Manolagas SC, Provvedini DM and Tsoukas CD (1985): Interactions of 1,25-dihydroxyvitamin D3 and the immune system. Mol Cell Endocrinol 43**:**113-122.

Masuda S, Kaufmann M, Byford V, Gao M, St-Arnaud R, Arabian A, Makin HL, Knutson JC, Strugnell S and Jones G (2004): Insights into Vitamin D metabolism using cyp24 over-expression and knockout systems in conjunction with liquid chromatography/mass spectrometry (LC/MS). J Steroid Biochem Mol Biol 89-90**:**149-153.

Masuda S, Byford V, Arabian A, Sakai Y, Demay MB, St-Arnaud R and Jones G (2005): Altered pharmacokinetics of 1alpha,25-dihydroxyvitamin D3 and 25-hydroxyvitamin D3 in the blood and tissues of the 25-hydroxyvitamin D-24-hydroxylase (Cyp24a1) null mouse. Endocrinology 146**:**825-834.

Mathiasen IS, Hansen CM, Foghsgaard L and Jaattela M (2001): Sensitization to TNF-induced apoptosis by 1,25-dihydroxy vitamin D(3) involves up-regulation of the TNF receptor 1 and cathepsin B. Int J Cancer 93**:**224-231.

Matkovits T and Christakos S (1995a): Variable in vivo regulation of rat vitamin D-dependent genes (osteopontin, Ca,Mg-adenosine triphosphatase, and 25-hydroxyvitamin D3 24-hydroxylase): implications for differing mechanisms of regulation and involvement of multiple factors. Endocrinology 136**:**3971-3982.

Matkovits T and Christakos S (1995b): Ligand occupancy is not required for vitamin D receptor and retinoid receptor-mediated transcriptional activation. Mol Endocrinol 9**:**232-242.

Matsuoka LY, Ide L, Wortsman J, MacLaughlin JA and Holick MF (1987): Sunscreens suppress cutaneous vitamin D3 synthesis. J Clin Endocrinol Metab 64**:**1165-1168.

Matsuoka LY, Wortsman J, Dannenberg MJ, Hollis BW, Lu Z and Holick MF (1992): Clothing prevents ultraviolet-B radiation-dependent photosynthesis of vitamin D3. J Clin Endocrinol Metab 75**:**1099-1103.

McCarthy TC, Li X and Sinal CJ (2005): Vitamin D receptor-dependent regulation of colon multidrug resistance-associated protein 3 gene expression by bile acids. J Biol Chem 280**:**23232-23242.

McCollum EV, Simmonds N, Becker JE and Shipley PG (1922): An experimental demonstration of the existence of a vitamin which promotes calcium deposition. J Biol Chem 53**:**293-298.

McDonnell DP, Mangelsdorf DJ, Pike JW, Haussler MR and O'Malley BW (1987): Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. Science 235**:**1214-1217.

McGaffin KR, Acktinson LE and Chrysogelos SA (2004): Growth and EGFR regulation in breast cancer cells by vitamin D and retinoid compounds. Breast Cancer Res Treat 86**:**55-73.

McKenna NJ and O'Malley BW (2002): Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 108**:**465-474.

McKenna NJ, Lanz RB and O'Malley BW (1999): Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 20**:**321-344.

McPherson SJ, Wang H, Jones ME, Pedersen J, Iismaa TP, Wreford N, Simpson ER and Risbridger GP (2001): Elevated androgens and prolactin in aromatase-deficient mice cause enlargement, but not malignancy, of the prostate gland. Endocrinology 142**:**2458-2467.

Mehta RG and Mehta RR (2002): Vitamin D and cancer. J Nutr Biochem 13**:**252-264.

Mellanby E (1919): An experimental investigation on rickets. Lancet 1**:**407-412.

Mesbah M, Nemere I, Papagerakis P, Nefussi JR, Orestes-Cardoso S, Nessmann C and Berdal A (2002): Expression of a 1,25-dihydroxyvitamin D3 membrane-associated rapid-response steroid

binding protein during human tooth and bone development and biomineralization. J Bone Miner Res 17:1588-1596.

Meyer ME, Gronemeyer H, Turcotte B, Bocquel MT, Tasset D and Chambon P (1989): Steroid hormone receptors compete for factors that mediate their enhancer function. Cell 57:433-442.

Miettinen S, Ahonen MH, Lou YR, Manninen T, Tuohimaa P, Syvala H and Ylikomi T (2004): Role of 24-hydroxylase in vitamin D3 growth response of OVCAR-3 ovarian cancer cells. Int J Cancer 108:367-373.

Miller GJ, Stapleton GE, Ferrara JA, Lucia MS, Pfister S, Hedlund TE and Upadhya P (1992): The human prostatic carcinoma cell line LNCaP expresses biologically active, specific receptors for 1 alpha,25-dihydroxyvitamin D3. Cancer Res 52:515-520.

Miller GJ, Stapleton GE, Hedlund TE and Moffat KA (1995): Vitamin D receptor expression, 24-hydroxylase activity, and inhibition of growth by 1alpha,25-dihydroxyvitamin D3 in seven human prostatic carcinoma cell lines. Clin Cancer Res 1:997-1003.

Miyamoto K, Kesterson RA, Yamamoto H, Taketani Y, Nishiwaki E, Tatsumi S, Inoue Y, Morita K, Takeda E and Pike JW (1997): Structural organization of the human vitamin D receptor chromosomal gene and its promoter. Mol Endocrinol 11:1165-1179.

Mizwicki MT and Norman AW (2003): Two key proteins of the vitamin D endocrine system come into crystal clear focus: comparison of the X-ray structures of the nuclear receptor for 1alpha,25(OH)2 vitamin D3, the plasma vitamin D binding protein, and their ligands. J Bone Miner Res 18:795-806.

Monkawa T, Yoshida T, Wakino S, Shinki T, Anazawa H, Deluca HF, Suda T, Hayashi M and Saruta T (1997): Molecular cloning of cDNA and genomic DNA for human 25-hydroxyvitamin D3 1 alpha-hydroxylase. Biochem Biophys Res Commun 239:527-533.

Monkawa T, Yoshida T, Hayashi M and Saruta T (2000): Identification of 25-hydroxyvitamin D3 1alpha-hydroxylase gene expression in macrophages. Kidney Int 58:559-568.

Morrison NA, Shine J, Fragonas JC, Verkest V, McMenemy ML and Eisman JA (1989): 1,25-dihydroxyvitamin D-responsive element and glucocorticoid repression in the osteocalcin gene. Science 246:1158-1161.

Morrison NA, Yeoman R, Kelly PJ and Eisman JA (1992): Contribution of trans-acting factor alleles to normal physiological variability: vitamin D receptor gene polymorphism and circulating osteocalcin. Proc Natl Acad Sci U S A 89:6665-6669.

Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN and Eisman JA (1994): Prediction of bone density from vitamin D receptor alleles. Nature 367:284-287.

Mueller MM and Fusenig NE (2004): Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 4:839-849.

Muller D, Hoenderop JG, Meij IC, van den Heuvel LP, Knoers NV, den Hollander AI, Eggert P, Garcia-Nieto V, Claverie-Martin F and Bindels RJ (2000): Molecular cloning, tissue distribution, and chromosomal mapping of the human epithelial Ca2+ channel (ECAC1). Genomics 67**:**48-53.

Murayama A, Takeyama K, Kitanaka S, Kodera Y, Hosoya T and Kato S (1998): The promoter of the human 25-hydroxyvitamin D3 1 alpha-hydroxylase gene confers positive and negative responsiveness to PTH, calcitonin, and 1 alpha,25(OH)2D3. Biochem Biophys Res Commun 249**:**11-16.

Murayama A, Takeyama K, Kitanaka S, Kodera Y, Kawaguchi Y, Hosoya T and Kato S (1999): Positive and negative regulations of the renal 25-hydroxyvitamin D3 1alpha-hydroxylase gene by parathyroid hormone, calcitonin, and 1alpha,25(OH)2D3 in intact animals. Endocrinology 140**:**2224-2231.

Murthy S and Weigel NL (2004): 1alpha,25-dihydroxyvitamin D3 induced growth inhibition of PC-3 prostate cancer cells requires an active transforming growth factor beta signaling pathway. Prostate 59**:**282-291.

Muto A, Kizaki M, Yamato K, Kawai Y, Kamata-Matsushita M, Ueno H, Ohguchi M, Nishihara T, Koeffler HP and Ikeda Y (1999): 1,25-Dihydroxyvitamin D3 induces differentiation of a retinoic acid-resistant acute promyelocytic leukemia cell line (UF-1) associated with expression of p21(WAF1/CIP1) and p27(KIP1). Blood 93**:**2225-2233.

Nagpal S, Na S and Rathnachalam R (2005): Noncalcemic actions of vitamin D receptor ligands. Endocr Rev 26**:**662-687.

Nakamura T, Kurokawa T and Orimo H (1987): Action of 24R, 25 dihydroxyvitamin D3 (24,25 (OH)2D3) on bone in vivo. In Vivo 1**:**313-317.

Nakamura T, Kurokawa T and Orimo H (1988): Increase of bone volume in vitamin D-repleted rats by massive administration of 24R,25(OH)2D3. Calcif Tissue Int 43**:**235-243.

Nakamura T, Kurokawa T and Orimo H (1989): Increased mechanical strength of the vitamin D-replete rat femur by the treatment with a large dose of 24R,25(OH)2D3. Bone 10**:**117-123.

Nakamura T, Suzuki K, Hirai T, Kurokawa T and Orimo H (1992): Increased bone volume and reduced bone turnover in vitamin D-replete rabbits by the administration of 24R,25-dihydroxyvitamin D3. Bone 13**:**229-236.

Nakayama T, Watanabe M, Yamanaka M, Hirokawa Y, Suzuki H, Ito H, Yatani R and Shiraishi T (2001): The role of epigenetic modifications in retinoic acid receptor beta2 gene expression in human prostate cancers. Lab Invest 81**:**1049-1057.

Negri-Cesi P, Poletti A, Colciago A, Magni P, Martini P and Motta M (1998): Presence of 5alpha-reductase isozymes and aromatase in human prostate cancer cells and in benign prostate hyperplastic tissue. Prostate 34**:**283-291.

Negri-Cesi P, Colciago A, Poletti A and Motta M (1999): 5alpha-reductase isozymes and aromatase are differentially expressed and active in the androgen-independent human prostate cancer cell lines DU145 and PC3. Prostate 41**:**224-232.

Nemere I (1999): 24,25-dihydroxyvitamin D3 suppresses the rapid actions of 1, 25-dihydroxyvitamin D3 and parathyroid hormone on calcium transport in chick intestine. J Bone Miner Res 14**:**1543-1549.

Nemere I, Dormanen MC, Hammond MW, Okamura WH and Norman AW (1994): Identification of a specific binding protein for 1 alpha,25-dihydroxyvitamin D3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. J Biol Chem 269**:**23750-23756.

Nemere I, Schwartz Z, Pedrozo H, Sylvia VL, Dean DD and Boyan BD (1998): Identification of a membrane receptor for 1,25-dihydroxyvitamin D3 which mediates rapid activation of protein kinase C. J Bone Miner Res 13**:**1353-1359.

Nemere I, Farach-Carson MC, Rohe B, Sterling TM, Norman AW, Boyan BD and Safford SE (2004): Ribozyme knockdown functionally links a 1,25(OH)2D3 membrane binding protein (1,25D3-MARRS) and phosphate uptake in intestinal cells. Proc Natl Acad Sci U S A 101**:**7392-7397.

Nevalainen MT, Valve EM, Makela SI, Blauer M, Tuohimaa PJ and Harkonen PL (1991): Estrogen and prolactin regulation of rat dorsal and lateral prostate in organ culture. Endocrinology 129**:**612-622.

Nevalainen MT, Harkonen PL, Valve EM, Ping W, Nurmi M and Martikainen PM (1993): Hormone regulation of human prostate in organ culture. Cancer Res 53**:**5199-5207.

Nguyen TM, Lieberherr M, Fritsch J, Guillozo H, Alvarez ML, Fitouri Z, Jehan F and Garabedian M (2004): The Rapid Effects of 1,25-Dihydroxyvitamin D3 Require the Vitamin D Receptor and Influence 24-Hydroxylase Activity: STUDIES IN HUMAN SKIN FIBROBLASTS BEARING VITAMIN D RECEPTOR MUTATIONS. J Biol Chem 279**:**7591-7597.

Nicke B, Riecken EO and Rosewicz S (1999): Induction of retinoic acid receptor beta mediates growth inhibition in retinoid resistant human colon carcinoma cells. Gut 45**:**51-57.

Nijenhuis T, Hoenderop JG, van der Kemp AW and Bindels RJ (2003): Localization and regulation of the epithelial Ca2+ channel TRPV6 in the kidney. J Am Soc Nephrol 14**:**2731-2740.

Nishimura A, Shinki T, Jin CH, Ohyama Y, Noshiro M, Okuda K and Suda T (1994): Regulation of messenger ribonucleic acid expression of 1 alpha,25-dihydroxyvitamin D3-24-hydroxylase in rat osteoblasts. Endocrinology 134**:**1794-1799.

Noda M, Vogel RL, Craig AM, Prahl J, DeLuca HF and Denhardt DT (1990): Identification of a DNA sequence responsible for binding of the 1,25- dihydroxyvitamin D3 receptor and 1,25-

dihydroxyvitamin D3 enhancement of mouse secreted phosphoprotein 1 (SPP-1 or osteopontin) gene expression. Proc Natl Acad Sci U S A 87**:**9995-9999.

Nomura AM, Stemmermann GN, Lee J, Kolonel LN, Chen TC, Turner A and Holick MF (1998): Serum vitamin D metabolite levels and the subsequent development of prostate cancer (Hawaii, United States). Cancer Causes Control 9**:**425-432.

Norman AW, Leathers V and Bishop JE (1983): Normal egg hatchability requires the simultaneous administration to the hen of 1 alpha,25-dihydroxycholecalciferol and 24R,25-dihydroxycholecalciferol. J Nutr 113**:**2505-2515.

Norman AW, Okamura WH, Farach-Carson MC, Allewaert K, Branisteanu D, Nemere I, Muralidharan KR and Bouillon R (1993): Structure-function studies of 1,25-dihydroxyvitamin D3 and the vitamin D endocrine system. 1,25-dihydroxy-pentadeuterio-previtamin D3 (as a 6-s-cis analog) stimulates nongenomic but not genomic biological responses. J Biol Chem 268**:**13811-13819.

Norman AW, Okamura WH, Bishop JE and Henry HL (2002): Update on biological actions of 1alpha,25(OH)2-vitamin D3 (rapid effects) and 24R,25(OH)2-vitamin D3. Mol Cell Endocrinol 197**:**1-13.

Nwankwo JO (2002): Anti-metastatic activities of all-trans retinoic acid, indole-3-carbinol and (+)-catechin in Dunning rat invasive prostate adenocarcinoma cells. Anticancer Res 22**:**4129-4135.

Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, Melsen F, Christensen EI and Willnow TE (1999): An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. Cell 96**:**507-515.

Oades GM, Dredge K, Kirby RS and Colston KW (2002): Vitamin D receptor-dependent antitumour effects of 1,25-dihydroxyvitamin D3 and two synthetic analogues in three in vivo models of prostate cancer. BJU Int 90**:**607-616.

Ohyama Y and Okuda K (1991): Isolation and characterization of a cytochrome P-450 from rat kidney mitochondria that catalyzes the 24-hydroxylation of 25-hydroxyvitamin D3. J Biol Chem 266**:**8690-8695.

Okano K, Usa T, Ohtsuru A, Tsukazaki T, Miyazaki Y, Yonekura A, Namba H, Shindoh H and Yamashita S (1999): Effect of 22-oxa-1,25-dihydroxyvitamin D3 on human thyroid cancer cell growth. Endocr J 46**:**243-252.

Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD and Cunha GR (1999): Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 59**:**5002-5011.

Omdahl JL, Morris HA and May BK (2002): Hydroxylase enzymes of the vitamin D pathway: expression, function, and regulation. Annu Rev Nutr 22**:**139-166.

Ono T, Tanaka H, Yamate T, Nagai Y, Nakamura T and Seino Y (1996): 24R,25-dihydroxyvitamin D3 promotes bone formation without causing excessive resorption in hypophosphatemic mice. Endocrinology 137**:**2633-2637.

Ornoy A, Goodwin D, Noff D and Edelstein S (1978): 24, 25-dihydroxyvitamin D is a metabolite of vitamin D essential for bone formation. Nature 276**:**517-519.

Osborn JL, Schwartz GG, Smith DC, Bahnson R, Day R and Trump DL (1995): Phase II trial of oral 1,25-dihydroxyvitamin D (calcitriol) in hormone refractory prostate cancer. Urol Oncol 1**:**195-198.

Palmer HG, Gonzalez-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, Quintanilla M, Cano A, de Herreros AG, Lafarga M and Munoz A (2001): Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. J Cell Biol 154**:**369-387.

Palmqvist P, Persson E, Conaway HH and Lerner UH (2002): IL-6, leukemia inhibitory factor, and oncostatin M stimulate bone resorption and regulate the expression of receptor activator of NF-kappa B ligand, osteoprotegerin, and receptor activator of NF-kappa B in mouse calvariae. J Immunol 169**:**3353-3362.

Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN and Goltzman D (2001): Targeted ablation of the 25-hydroxyvitamin D 1alpha -hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. Proc Natl Acad Sci U S A 98**:**7498-7503.

Panda DK, Miao D, Bolivar I, Li J, Huo R, Hendy GN and Goltzman D (2004): Inactivation of the 25-hydroxyvitamin D 1alpha-hydroxylase and vitamin D receptor demonstrates independent and interdependent effects of calcium and vitamin D on skeletal and mineral homeostasis. J Biol Chem 279**:**16754-16766.

Parfitt AM, Mathews CH, Brommage R, Jarnagin K and DeLuca HF (1984): Calcitriol but no other metabolite of vitamin D is essential for normal bone growth and development in the rat. J Clin Invest 73**:**576-586.

Pavlin D, Bedalov A, Kronenberg MS, Kream BE, Rowe DW, Smith CL, Pike JW and Lichtler AC (1994): Analysis of regulatory regions in the COL1A1 gene responsible for 1,25-dihydroxyvitamin D3-mediated transcriptional repression in osteoblastic cells. J Cell Biochem 56**:**490-501.

Pedigo N, Zhang H, Koszewski NJ and Kaetzel DM (2003): A 5'-distal element mediates vitamin D-inducibility of PDGF-A gene transcription. Growth Factors 21**:**151-160.

Pedrozo HA, Schwartz Z, Rimes S, Sylvia VL, Nemere I, Posner GH, Dean DD and Boyan BD (1999): Physiological importance of the 1,25(OH)2D3 membrane receptor and evidence for a membrane receptor specific for 24,25(OH)2D3. J Bone Miner Res 14**:**856-867.

Peehl DM and Sellers RG (1997): Induction of smooth muscle cell phenotype in cultured human prostatic stromal cells. Exp Cell Res 232**:**208-215.

Peehl DM, Wong ST and Stamey TA (1993): Vitamin A regulates proliferation and differentiation of human prostatic epithelial cells. Prostate 23**:**69-78.

Peehl DM, Leung GK and Wong ST (1994): Keratin expression: a measure of phenotypic modulation of human prostatic epithelial cells by growth inhibitory factors. Cell Tissue Res 277**:**11-18.

Peehl DM, Seto E and Feldman D (2001): Rationale for combination ketoconazole/ vitamin D treatment of prostate cancer. Urology 58**:**123-126.

Peehl DM, Seto E, Hsu JY and Feldman D (2002): Preclinical activity of ketoconazole in combination with calcitriol or the vitamin D analogue EB 1089 in prostate cancer cells. J Urol 168**:**1583-1588.

Peng JB, Chen XZ, Berger UV, Weremowicz S, Morton CC, Vassilev PM, Brown EM and Hediger MA (2000): Human calcium transport protein CaT1. Biochem Biophys Res Commun 278**:**326-332.

Peng L, Malloy PJ and Feldman D (2004): Identification of a functional vitamin D response element in the human insulin-like growth factor binding protein-3 promoter. Mol Endocrinol 18**:**1109-1119.

Penna G and Adorini L (2000): 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. J Immunol 164**:**2405-2411.

Pepper C, Thomas A, Hoy T, Milligan D, Bentley P and Fegan C (2003): The vitamin D3 analog EB1089 induces apoptosis via a p53-independent mechanism involving p38 MAP kinase activation and suppression of ERK activity in B-cell chronic lymphocytic leukemia cells in vitro. Blood 101**:**2454-2460.

Pfaffl MW (2001): A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29**:**E45-45.

Polly P, Carlberg C, Eisman JA and Morrison NA (1996): Identification of a vitamin D3 response element in the fibronectin gene that is bound by a vitamin D3 receptor homodimer. J Cell Biochem 60**:**322-333.

Polly P, Herdick M, Moehren U, Baniahmad A, Heinzel T and Carlberg C (2000): VDR-Alien: a novel, DNA-selective vitamin D(3) receptor-corepressor partnership. Faseb J 14**:**1455-1463.

Ponchon G and DeLuca HF (1969): The role of the liver in the metabolism of vitamin D. J Clin Invest 48**:**1273-1279.

Ponchon G, Kennan AL and DeLuca HF (1969): "Activation" of vitamin D by the liver. J Clin Invest 48**:**2032-2037.

Prehn RT (1999): On the prevention and therapy of prostate cancer by androgen administration. Cancer Res 59**:**4161-4164.

Prufer K, Racz A, Lin GC and Barsony J (2000): Dimerization with retinoid X receptors promotes nuclear localization and subnuclear targeting of vitamin D receptors. J Biol Chem 275**:**41114-41123.

Puthier D, Bataille R, Barille S, Mellerin MP, Harousseau JL, Ponzio A, Robillard N, Wijdenes J and Amiot M (1996): Myeloma cell growth arrest, apoptosis, and interleukin-6 receptor modulation induced by EB1089, a vitamin D3 derivative, alone or in association with dexamethasone. Blood 88**:**4659-4666.

Quelo I, Machuca I and Jurdic P (1998): Identification of a vitamin D response element in the proximal promoter of the chicken carbonic anhydrase II gene. J Biol Chem 273**:**10638-10646.

Rachez C and Freedman LP (2000): Mechanisms of gene regulation by vitamin D(3) receptor: a network of coactivator interactions. Gene 246**:**9-21.

Rastinejad F (2001): Retinoid X receptor and its partners in the nuclear receptor family. Curr Opin Struct Biol 11**:**33-38.

Raval-Pandya M, Freedman LP, Li H and Christakos S (1998): Thyroid hormone receptor does not heterodimerize with the vitamin D receptor but represses vitamin D receptor-mediated transactivation. Mol Endocrinol 12**:**1367-1379.

Raval-Pandya M, Dhawan P, Barletta F and Christakos S (2001): YY1 represses vitamin D receptor-mediated 25-hydroxyvitamin D(3)24-hydroxylase transcription: relief of repression by CREB-binding protein. Mol Endocrinol 15**:**1035-1046.

Reinhardt TA and Horst RL (1989): Ketoconazole inhibits self-induced metabolism of 1,25-dihydroxyvitamin D3 and amplifies 1,25-dihydroxyvitamin D3 receptor up-regulation in rat osteosarcoma cells. Arch Biochem Biophys 272**:**459-465.

Reinhardt TA, Horst RL, Orf JW and Hollis BW (1984): A microassay for 1,25-dihydroxyvitamin D not requiring high performance liquid chromatography: application to clinical studies. J Clin Endocrinol Metab 58**:**91-98.

Ren S, Nguyen L, Wu S, Encinas C, Adams JS and Hewison M (2005): Alternative splicing of vitamin D-24-hydroxylase: a novel mechanism for the regulation of extrarenal 1,25-dihydroxyvitamin D synthesis. J Biol Chem 280**:**20604-20611.

Risbridger GP, Bianco JJ, Ellem SJ and McPherson SJ (2003): Oestrogens and prostate cancer. Endocr Relat Cancer 10**:**187-191.

Rosen H, Reshef A, Maeda N, Lippoldt A, Shpizen S, Triger L, Eggertsen G, Bjorkhem I and Leitersdorf E (1998): Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. J Biol Chem 273**:**14805-14812.

Rowan BG, Weigel NL and O'Malley BW (2000a): Phosphorylation of steroid receptor coactivator-1. Identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. J Biol Chem 275**:**4475-4483.

Rowan BG, Garrison N, Weigel NL and O'Malley BW (2000b): 8-Bromo-cyclic AMP induces phosphorylation of two sites in SRC-1 that facilitate ligand-independent activation of the chicken progesterone receptor and are critical for functional cooperation between SRC-1 and CREB binding protein. Mol Cell Biol 20**:**8720-8730.

Royuela M, de Miguel MP, Bethencourt FR, Sanchez-Chapado M, Fraile B, Arenas MI and Paniagua R (2001): Estrogen receptors alpha and beta in the normal, hyperplastic and carcinomatous human prostate. J Endocrinol 168**:**447-454.

Ruijter E, van de Kaa C, Miller G, Ruiter D, Debruyne F and Schalken J (1999): Molecular genetics and epidemiology of prostate carcinoma. Endocr Rev 20**:**22-45.

Sakaki T, Sawada N, Nonaka Y, Ohyama Y and Inouye K (1999a): Metabolic studies using recombinant escherichia coli cells producing rat mitochondrial CYP24 CYP24 can convert 1alpha,25-dihydroxyvitamin D3 to calcitroic acid. Eur J Biochem 262**:**43-48.

Sakaki T, Sawada N, Takeyama K, Kato S and Inouye K (1999b): Enzymatic properties of mouse 25-hydroxyvitamin D3 1 alpha-hydroxylase expressed in Escherichia coli. Eur J Biochem 259**:**731-738.

Sakaki T, Sawada N, Komai K, Shiozawa S, Yamada S, Yamamoto K, Ohyama Y and Inouye K (2000): Dual metabolic pathway of 25-hydroxyvitamin D3 catalyzed by human CYP24. Eur J Biochem 267**:**6158-6165.

Salih FM (2004): Effect of clothing varieties on solar photosynthesis of previtamin D3: an in vitro study. Photodermatol Photoimmunol Photomed 20**:**53-58.

Sawada N, Sakaki T, Kitanaka S, Takeyama K, Kato S and Inouye K (1999): Enzymatic properties of human 25-hydroxyvitamin D3 1alpha-hydroxylase coexpression with adrenodoxin and NADPH-adrenodoxin reductase in Escherichia coli. Eur J Biochem 265**:**950-956.

Scaglione-Sewell BA, Bissonnette M, Skarosi S, Abraham C and Brasitus TA (2000): A vitamin D3 analog induces a G1-phase arrest in CaCo-2 cells by inhibiting cdk2 and cdk6: roles of cyclin E, p21Waf1, and p27Kip1. Endocrinology 141**:**3931-3939.

Schmid C, Schlapfer I, Gosteli-Peter MA, Hauri C, Froesch ER and Zapf J (1996): 1 alpha,25-dihydroxyvitamin D3 increases IGF binding protein-5 expression in cultured osteoblasts. FEBS Lett 392**:**21-24.

Schneider SM, Offterdinger M, Huber H and Grunt TW (2000): Activation of retinoic acid receptor alpha is sufficient for full induction of retinoid responses in SK-BR-3 and T47D human breast cancer cells. Cancer Res 60**:**5479-5487.

Schuessler M, Astecker N, Herzig G, Vorisek G and Schuster I (2001): Skin is an autonomous organ in synthesis, two-step activation and degradation of vitamin D(3): CYP27 in epidermis completes the set of essential vitamin D(3)-hydroxylases. Steroids 66**:**399-408.

Schuster I, Egger H, Astecker N, Herzig G, Schussler M and Vorisek G (2001a): Selective inhibitors of CYP24: mechanistic tools to explore vitamin D metabolism in human keratinocytes. Steroids 66**:**451-462.

Schuster I, Egger H, Bikle D, Herzig G, Reddy GS, Stuetz A, Stuetz P and Vorisek G (2001b): Selective inhibition of vitamin D hydroxylases in human keratinocytes. Steroids 66**:**409-422.

Schwartz GG and Hulka BS (1990): Is vitamin D deficiency a risk factor for prostate cancer? (Hypothesis). Anticancer Res 10**:**1307-1311.

Schwartz GG, Hill CC, Oeler TA, Becich MJ and Bahnson RR (1995a): 1,25-Dihydroxy-16-ene-23-yne-vitamin D3 and prostate cancer cell proliferation in vivo. Urology 46**:**365-369.

Schwartz Z, Dean DD, Walton JK, Brooks BP and Boyan BD (1995b): Treatment of resting zone chondrocytes with 24,25-dihydroxyvitamin D3 [24,25-(OH)2D3] induces differentiation into a 1,25-(OH)2D3-responsive phenotype characteristic of growth zone chondrocytes. Endocrinology 136**:**402-411.

Schwartz GG, Wang MH, Zang M, Singh RK and Siegal GP (1997): 1 alpha,25-Dihydroxyvitamin D (calcitriol) inhibits the invasiveness of human prostate cancer cells. Cancer Epidemiol Biomarkers Prev 6**:**727-732.

Schwartz GG, Whitlatch LW, Chen TC, Lokeshwar BL and Holick MF (1998): Human prostate cells synthesize 1,25-dihydroxyvitamin D3 from 25- hydroxyvitamin D3. Cancer Epidemiol Biomarkers Prev 7**:**391-395.

Schweikert HU, Milewich L and Wilson JD (1976): Aromatization of androstenedione by cultured human fibroblasts. J Clin Endocrinol Metab 43**:**785-795.

Segersten U, Correa P, Hewison M, Hellman P, Dralle H, Carling T, Akerstrom G and Westin G (2002): 25-hydroxyvitamin D(3)-1alpha-hydroxylase expression in normal and pathological parathyroid glands. J Clin Endocrinol Metab 87**:**2967-2972.

Seo EG and Norman AW (1997): Three-fold induction of renal 25-hydroxyvitamin D3-24-hydroxylase activity and increased serum 24,25-dihydroxyvitamin D3 levels are correlated with the healing process after chick tibial fracture. J Bone Miner Res 12**:**598-606.

Seo EG, Einhorn TA and Norman AW (1997): 24R,25-dihydroxyvitamin D3: an essential vitamin D3 metabolite for both normal bone integrity and healing of tibial fracture in chicks. Endocrinology 138**:**3864-3872.

Shaffer PL, McDonnell DP and Gewirth DT (2005): Characterization of Transcriptional Activation and DNA-Binding Functions in the Hinge Region of the Vitamin D Receptor. Biochemistry 44**:**2678-2685.

Shang Y, Baumrucker CR and Green MH (1999): Signal relay by retinoic acid receptors alpha and beta in the retinoic acid-induced expression of insulin-like growth factor-binding protein-3 in breast cancer cells. J Biol Chem 274**:**18005-18010.

Shinki T, Jin CH, Nishimura A, Nagai Y, Ohyama Y, Noshiro M, Okuda K and Suda T (1992): Parathyroid hormone inhibits 25-hydroxyvitamin D3-24-hydroxylase mRNA expression stimulated by 1 alpha,25-dihydroxyvitamin D3 in rat kidney but not in intestine. J Biol Chem 267**:**13757-13762.

Simboli-Campbell M, Narvaez CJ, Tenniswood M and Welsh J (1996): 1,25-Dihydroxyvitamin D3 induces morphological and biochemical markers of apoptosis in MCF-7 breast cancer cells. J Steroid Biochem Mol Biol 58**:**367-376.

Sinkkonen L, Malinen M, Saavalainen K, Vaisanen S and Carlberg C (2005): Regulation of the human cyclin C gene via multiple vitamin D3-responsive regions in its promoter. Nucleic Acids Res 33**:**2440-2451.

Sirchia SM, Ren M, Pili R, Sironi E, Somenzi G, Ghidoni R, Toma S, Nicolo G and Sacchi N (2002): Endogenous reactivation of the RARbeta2 tumor suppressor gene epigenetically silenced in breast cancer. Cancer Res 62**:**2455-2461.

Skorija K, Cox M, Sisk JM, Dowd DR, MacDonald PN, Thompson CC and Demay MB (2005): Ligand-independent actions of the vitamin D receptor maintain hair follicle homeostasis. Mol Endocrinol 19**:**855-862.

Skowronski RJ, Peehl DM and Feldman D (1993): Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D3 receptors and actions in human prostate cancer cell lines. Endocrinology 132**:**1952-1960.

Skowronski RJ, Peehl DM and Feldman D (1995): Actions of vitamin D3, analogs on human prostate cancer cell lines: comparison with 1,25-dihydroxyvitamin D3. Endocrinology 136**:**20-26.

Smith DC, Johnson CS, Freeman CC, Muindi J, Wilson JW and Trump DL (1999): A Phase I trial of calcitriol (1,25-dihydroxycholecalciferol) in patients with advanced malignancy. Clin Cancer Res 5**:**1339-1345.

Solomon C, White JH and Kremer R (1999): Mitogen-activated protein kinase inhibits 1,25-dihydroxyvitamin D3- dependent signal transduction by phosphorylating human retinoid X receptor alpha. J Clin Invest 103**:**1729-1735.

Sonnenschein C, Olea N, Pasanen ME and Soto AM (1989): Negative controls of cell proliferation: human prostate cancer cells and androgens. Cancer Res 49**:**3474-3481.

St-Arnaud R, Messerlian S, Moir JM, Omdahl JL and Glorieux FH (1997): The 25-hydroxyvitamin D 1-alpha-hydroxylase gene maps to the pseudovitamin D-deficiency rickets (PDDR) disease locus. J Bone Miner Res 12**:**1552-1559.

St-Arnaud R, Arabian A, Travers R, Barletta F, Raval-Pandya M, Chapin K, Depovere J, Mathieu C, Christakos S, Demay MB and Glorieux FH (2000): Deficient mineralization of intramembranous bone in vitamin D-24-hydroxylase-ablated mice is due to elevated 1,25-dihydroxyvitamin D and not to the absence of 24,25-dihydroxyvitamin D. Endocrinology 141**:**2658-2666.

Stattin P, Bylund A, Rinaldi S, Biessy C, Dechaud H, Stenman UH, Egevad L, Riboli E, Hallmans G and Kaaks R (2000): Plasma insulin-like growth factor-I, insulin-like growth factor-binding proteins, and prostate cancer risk: a prospective study. J Natl Cancer Inst 92**:**1910-1917.

Stattin P, Lumme S, Tenkanen L, Alfthan H, Jellum E, Hallmans G, Thoresen S, Hakulinen T, Luostarinen T, Lehtinen M, Dillner J, Stenman UH and Hakama M (2004): High levels of circulating testosterone are not associated with increased prostate cancer risk: a pooled prospective study. Int J Cancer 108**:**418-424.

Suarez F, Zeghoud F, Rossignol C, Walrant O and Garabedian M (1997): Association between vitamin D receptor gene polymorphism and sex-dependent growth during the first two years of life. J Clin Endocrinol Metab 82**:**2966-2970.

Sung V and Feldman D (2000): 1,25-Dihydroxyvitamin D3 decreases human prostate cancer cell adhesion and migration. Mol Cell Endocrinol 164**:**133-143.

Sunn KL, Cock TA, Crofts LA, Eisman JA and Gardiner EM (2001): Novel N-terminal variant of human VDR. Mol Endocrinol 15**:**1599-1609.

Swamy N, Chen TC, Peleg S, Dhawan P, Christakos S, Stewart LV, Weigel NL, Mehta RG, Holick MF and Ray R (2004): Inhibition of proliferation and induction of apoptosis by 25-hydroxyvitamin D3-3beta-(2)-Bromoacetate, a nontoxic and vitamin D receptor-alkylating analog of 25-hydroxyvitamin D3 in prostate cancer cells. Clin Cancer Res 10**:**8018-8027.

Szabo A, Merke J, Beier E, Mall G and Ritz E (1989): 1,25(OH)2 vitamin D3 inhibits parathyroid cell proliferation in experimental uremia. Kidney Int 35**:**1049-1056.

Tagami T, Lutz WH, Kumar R and Jameson JL (1998): The interaction of the vitamin D receptor with nuclear receptor corepressors and coactivators. Biochem Biophys Res Commun 253**:**358-363.

Tahka KM, Zhuang YH, Tahka S and Tuohimaa P (1997): Photoperiod-induced changes in androgen receptor expression in testes and accessory sex glands of the bank vole, Clethrionomys glareolus. Biol Reprod 56**:**898-908.

Taketani Y, Segawa H, Chikamori M, Morita K, Tanaka K, Kido S, Yamamoto H, Iemori Y, Tatsumi S, Tsugawa N, Okano T, Kobayashi T, Miyamoto K and Takeda E (1998): Regulation of type II renal Na+-dependent inorganic phosphate transporters by 1,25-dihydroxyvitamin D3. Identification of a vitamin D-responsive element in the human NAPi-3 gene. J Biol Chem 273**:**14575-14581.

Takeuchi K and Guggino SE (1996): 24R,25-(OH)2 vitamin D3 inhibits 1alpha,25-(OH)2 vitamin D3 and testosterone potentiation of calcium channels in osteosarcoma cells. J Biol Chem 271**:**33335-33343.

Tangpricha V, Flanagan JN, Whitlatch LW, Tseng CC, Chen TC, Holt PR, Lipkin MS and Holick MF (2001): 25-hydroxyvitamin D-1alpha-hydroxylase in normal and malignant colon tissue. Lancet 357**:**1673-1674.

Tangrea J, Helzlsouer K, Pietinen P, Taylor P, Hollis B, Virtamo J and Albanes D (1997): Serum levels of vitamin D metabolites and the subsequent risk of colon and rectal cancer in Finnish men. Cancer Causes Control 8**:**615-625.

Tanner MM, Tirkkonen M, Kallioniemi A, Holli K, Collins C, Kowbel D, Gray JW, Kallioniemi OP and Isola J (1995): Amplification of chromosomal region 20q13 in invasive breast cancer: prognostic implications. Clin Cancer Res 1**:**1455-1461.

Tanner MM, Grenman S, Koul A, Johannsson O, Meltzer P, Pejovic T, Borg A and Isola JJ (2000): Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer. Clin Cancer Res 6**:**1833-1839.

Tao C, Yu T, Garnett S, Briody J, Knight J, Woodhead H and Cowell CT (1998): Vitamin D receptor alleles predict growth and bone density in girls. Arch Dis Child 79**:**488-493; discussion 493-484.

Theodoropoulos C, Demers C, Mirshahi A and Gascon-Barre M (2001): 1,25-Dihydroxyvitamin D(3) downregulates the rat intestinal vitamin D(3)-25-hydroxylase CYP27A. Am J Physiol Endocrinol Metab 281**:**E315-325.

Theodoropoulos C, Demers C, Delvin E, Menard D and Gascon-Barre M (2003): Calcitriol regulates the expression of the genes encoding the three key vitamin D3 hydroxylases and the drug-metabolizing enzyme CYP3A4 in the human fetal intestine. Clin Endocrinol (Oxf) 58**:**489-499.

Thompson PD, Jurutka PW, Whitfield GK, Myskowski SM, Eichhorst KR, Dominguez CE, Haussler CA and Haussler MR (2002): Liganded VDR induces CYP3A4 in small intestinal and colon cancer cells via DR3 and ER6 vitamin D responsive elements. Biochem Biophys Res Commun 299**:**730-738.

Tlsty TD and Hein PW (2001): Know thy neighbor: stromal cells can contribute oncogenic signals. Curr Opin Genet Dev 11**:**54-59.

Tolon RM, Castillo AI, Jimenez-Lara AM and Aranda A (2000): Association with Ets-1 causes ligand- and AF2-independent activation of nuclear receptors. Mol Cell Biol 20**:**8793-8802.

Townsend K, Banwell CM, Guy M, Colston KW, Mansi JL, Stewart PM, Campbell MJ and Hewison M (2005): Autocrine Metabolism of Vitamin D in Normal and Malignant Breast Tissue. Clin Cancer Res 11**:**3579-3586.

Tsuchiya Y, Matsuo N, Cho H, Kumagai M, Yasaka A, Suda T, Orimo H and Shiraki M (1980): An unusual form of vitamin D-dependent rickets in a child: alopecia and marked end-organ hyposensitivity to biologically active vitamin D. J Clin Endocrinol Metab 51**:**685-690.

Tuohimaa P, Tenkanen L, Ahonen M, Lumme S, Jellum E, Hallmans G, Stattin P, Harvei S, Hakulinen T, Luostarinen T, Dillner J, Lehtinen M and Hakama M (2004): Both high and low levels of blood vitamin D are associated with a higher prostate cancer risk: a longitudinal, nested case-control study in the Nordic countries. Int J Cancer 108**:**104-108.

Turnbull H, Trafford DJ and Makin HL (1982): A rapid and simple method for the measurement of plasma 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 using Sep-Pak C18 cartridges and a single high-performance liquid chromatographic step. Clin Chim Acta 120**:**65-76.

Uitterlinden AG, Weel AE, Burger H, Fang Y, van Duijn CM, Hofman A, van Leeuwen JP and Pols HA (2001): Interaction between the vitamin D receptor gene and collagen type Ialpha1 gene in susceptibility for fracture. J Bone Miner Res 16**:**379-385.

Umemiya H, Kagechika H, Fukasawa H, Kawachi E, Ebisawa M, Hashimoto Y, Eisenmann G, Erb C, Pornon A, Chambon P, Gronemeyer H and Shudo K (1997): Action mechanism of retinoid-synergistic dibenzodiazepines. Biochem Biophys Res Commun 233**:**121-125.

Usheva A and Shenk T (1994): TATA-binding protein-independent initiation: YY1, TFIIB, and RNA polymerase II direct basal transcription on supercoiled template DNA. Cell 76**:**1115-1121.

Van Baal J, Yu A, Hartog A, Fransen JA, Willems PH, Lytton J and Bindels RJ (1996): Localization and regulation by vitamin D of calcium transport proteins in rabbit cortical collecting system. Am J Physiol 271**:**F985-993.

Van Cromphaut SJ, Dewerchin M, Hoenderop JG, Stockmans I, Van Herck E, Kato S, Bindels RJ, Collen D, Carmeliet P, Bouillon R and Carmeliet G (2001): Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. Proc Natl Acad Sci U S A 98**:**13324-13329.

Veldscholte J, Berrevoets CA, Ris-Stalpers C, Kuiper GG, Jenster G, Trapman J, Brinkmann AO and Mulder E (1992): The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. J Steroid Biochem Mol Biol 41**:**665-669.

Verlinden L, Verstuyf A, Convents R, Marcelis S, Van Camp M and Bouillon R (1998): Action of 1,25(OH)2D3 on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells. Mol Cell Endocrinol 142**:**57-65.

Verma AK, Filoteo AG, Stanford DR, Wieben ED, Penniston JT, Strehler EE, Fischer R, Heim R, Vogel G, Mathews S and et al. (1988): Complete primary structure of a human plasma membrane Ca2+ pump. J Biol Chem 263**:**14152-14159.

Vidal M, Ramana CV and Dusso AS (2002): Stat1-vitamin D receptor interactions antagonize 1,25-dihydroxyvitamin D transcriptional activity and enhance stat1-mediated transcription. Mol Cell Biol 22**:**2777-2787.

Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, Palotie A, Tammela T, Isola J and Kallioniemi OP (1995): In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 9**:**401-406.

Wali RK, Kong J, Sitrin MD, Bissonnette M and Li YC (2003): Vitamin D receptor is not required for the rapid actions of 1,25-dihydroxyvitamin D3 to increase intracellular calcium and activate protein kinase C in mouse osteoblasts. J Cell Biochem 88**:**794-801.

Walters MR (1992): Newly identified actions of the vitamin D endocrine system. Endocr Rev 13**:**719-764.

Walters MR, Ilenchuk TT and Claycomb WC (1987): 1,25-Dihydroxyvitamin D3 stimulates 45Ca2+ uptake by cultured adult rat ventricular cardiac muscle cells. J Biol Chem 262**:**2536-2541.

Wang Q and Wieder R (2004): All-trans retinoic acid potentiates Taxotere-induced cell death mediated by Jun N-terminal kinase in breast cancer cells. Oncogene 23**:**426-433.

Wang QM, Jones JB and Studzinski GP (1996): Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 cells. Cancer Res 56**:**264-267.

Wang Q, Yang W, Uytingco MS, Christakos S and Wieder R (2000): 1,25-Dihydroxyvitamin D3 and all-trans-retinoic acid sensitize breast cancer cells to chemotherapy-induced cell death. Cancer Res 60**:**2040-2048.

Wang TT, Tavera-Mendoza LE, Laperriere D, Libby E, MacLeod NB, Nagai Y, Bourdeau V, Konstorum A, Lallemant B, Zhang R, Mader S and White JH (2005): Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. Mol Endocrinol 19**:**2685-2695.

Weaver CM and Fleet JC (2004): Vitamin D requirements: current and future. Am J Clin Nutr 80**:**1735S-1739S.

Webb AR, Kline L and Holick MF (1988): Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin. J Clin Endocrinol Metab 67**:**373-378.

Weihua Z, Warner M and Gustafsson JA (2002): Estrogen receptor beta in the prostate. Mol Cell Endocrinol 193**:**1-5.

White P and Cooke N (2000): The multifunctional properties and characteristics of vitamin D-binding protein. Trends Endocrinol Metab 11**:**320-327.

Wiese RJ, Uhland-Smith A, Ross TK, Prahl JM and DeLuca HF (1992): Up-regulation of the vitamin D receptor in response to 1,25-dihydroxyvitamin D3 results from ligand-induced stabilization. J Biol Chem 267**:**20082-20086.

Wolter H, Gottfried HW and Mattfeldt T (2002): Genetic changes in stage pT2N0 prostate cancer studied by comparative genomic hybridization. BJU Int 89**:**310-316.

Wu Y, Craig TA, Lutz WH and Kumar R (1999): Identification of 1 alpha,25-dihydroxyvitamin D3 response elements in the human transforming growth factor beta 2 gene. Biochemistry 38**:**2654-2660.

Wu S, Ren S, Chen H, Chun RF, Gacad MA and Adams JS (2000): Intracellular vitamin D binding proteins: novel facilitators of vitamin D-directed transactivation. Mol Endocrinol 14**:**1387-1397.

Wu S, Chun R, Gacad MA, Ren S, Chen H and Adams JS (2002): Regulation of 1,25-dihydroxyvitamin d synthesis by intracellular vitamin d binding protein-1. Endocrinology 143**:**4135.

Xie Z and Bikle DD (1997): Cloning of the human phospholipase C-gamma1 promoter and identification of a DR6-type vitamin D-responsive element. J Biol Chem 272**:**6573-6577.

Xie Z and Bikle DD (2001): The role of phospholipase C-gamma1 in 1alpha,25-dihydroxyvitamin D(3) regulated keratinocyte differentiation. Steroids 66**:**339-345.

Xie Z, Munson SJ, Huang N, Portale AA, Miller WL and Bikle DD (2002): The mechanism of 1,25-dihydroxyvitamin D(3) autoregulation in keratinocytes. J Biol Chem 277**:**36987-36990.

Yamato H, Okazaki R, Ishii T, Ogata E, Sato T, Kumegawa M, Akaogi K, Taniguchi N and Matsumoto T (1993): Effect of 24R,25-dihydroxyvitamin D3 on the formation and function of osteoclastic cells. Calcif Tissue Int 52**:**255-260.

Yang ES and Burnstein KL (2003): Vitamin D inhibits G1 to S progression in LNCaP prostate cancer cells through p27Kip1 stabilization and Cdk2 mislocalization to the cytoplasm. J Biol Chem 278**:**46862-46868.

Ylikomi T, Laaksi I, Lou YR, Martikainen P, Miettinen S, Pennanen P, Purmonen S, Syvälä H, Vienonen A and Tuohimaa P (2002): Antiproliferative action of vitamin D. Vitam Horm 64**:**357-406.

Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T and Kato S (1997): Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat Genet 16**:**391-396.

Young MV, Schwartz GG, Wang L, Jamieson DP, Whitlatch LW, Flanagan JN, Lokeshwar BL, Holick MF and Chen TC (2004): The prostate 25-hydroxyvitamin D-1 alpha-hydroxylase is not

influenced by parathyroid hormone and calcium: implications for prostate cancer chemoprevention by vitamin D. Carcinogenesis 25:967-971.

Zanello LP and Norman AW (2004): Rapid modulation of osteoblast ion channel responses by 1alpha,25(OH)2-vitamin D3 requires the presence of a functional vitamin D nuclear receptor. Proc Natl Acad Sci U S A 101:1589-1594.

Zehnder D, Bland R, Walker EA, Bradwell AR, Howie AJ, Hewison M and Stewart PM (1999): Expression of 25-hydroxyvitamin D3-1alpha-hydroxylase in the human kidney. J Am Soc Nephrol 10:2465-2473.

Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM and Hewison M (2001): Extrarenal expression of 25-hydroxyvitamin d(3)-1 alpha-hydroxylase. J Clin Endocrinol Metab 86:888-894.

Zehnder D, Bland R, Chana RS, Wheeler DC, Howie AJ, Williams MC, Stewart PM and Hewison M (2002a): Synthesis of 1,25-dihydroxyvitamin D(3) by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion. J Am Soc Nephrol 13:621-629.

Zehnder D, Evans KN, Kilby MD, Bulmer JN, Innes BA, Stewart PM and Hewison M (2002b): The ontogeny of 25-hydroxyvitamin D(3) 1alpha-hydroxylase expression in human placenta and decidua. Am J Pathol 161:105-114.

Zhang XK, Liu Y, Lee MO and Pfahl M (1994): A specific defect in the retinoic acid response associated with human lung cancer cell lines. Cancer Res 54:5663-5669.

Zhang MY, Wang X, Wang JT, Compagnone NA, Mellon SH, Olson JL, Tenenhouse HS, Miller WL and Portale AA (2002): Dietary phosphorus transcriptionally regulates 25-hydroxyvitamin D-1alpha-hydroxylase gene expression in the proximal renal tubule. Endocrinology 143:587-595.

Zhao XY, Ly LH, Peehl DM and Feldman D (1997): 1alpha,25-dihydroxyvitamin D3 actions in LNCaP human prostate cancer cells are androgen-dependent. Endocrinology 138:3290-3298.

Zhao XY, Peehl DM, Navone NM and Feldman D (2000): 1alpha,25-dihydroxyvitamin D3 inhibits prostate cancer cell growth by androgen-dependent and androgen-independent mechanisms. Endocrinology 141:2548-2556.

Zhuang SH and Burnstein KL (1998): Antiproliferative effect of 1alpha,25-dihydroxyvitamin D3 in human prostate cancer cell line LNCaP involves reduction of cyclin-dependent kinase 2 activity and persistent G1 accumulation. Endocrinology 139:1197-1207.

Zhuang SH, Schwartz GG, Cameron D and Burnstein KL (1997): Vitamin D receptor content and transcriptional activity do not fully predict antiproliferative effects of vitamin D in human prostate cancer cell lines. Mol Cell Endocrinol 126:83-90.

Zierold C, Mings JA and DeLuca HF (2001): Parathyroid hormone regulates 25-hydroxyvitamin D(3)-24-hydroxylase mRNA by altering its stability. Proc Natl Acad Sci U S A 98**:**13572-13576.

Zierold C, Mings JA and DeLuca HF (2003): Regulation of 25-hydroxyvitamin D3-24-hydroxylase mRNA by 1,25-dihydroxyvitamin D3 and parathyroid hormone. J Cell Biochem 88**:**234-237.

Zierold C, Mings JA, Prahl JM, Reinholz GG and DeLuca HF (2002): Protein synthesis is required for optimal induction of 25-hydroxyvitamin D(3)-24-hydroxylase, osteocalcin, and osteopontin mRNA by 1,25-dihydroxyvitamin D(3). Arch Biochem Biophys 404**:**18-24.

Zittermann A (2003): Vitamin D in preventive medicine: are we ignoring the evidence? Br J Nutr 89**:**552-572.

ORIGINAL COMMUNICATIONS

ERRATA

There are two errors in the original communication III:

Page 1976, in the caption of Fig. 1, "$P < 0.05$ (ns)" should read "$P > 0.05$ (ns)";

Page 1978, in the caption of Fig. 3, "$P < 0.05$ (ns)" should read "$P > 0.05$ (ns)".