Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 92 (2004) 131–141
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Molecular activity of 1,25-dihydroxyvitamin D_3 in primary cultures of human prostatic epithelial cells revealed by cDNA microarray analysis

Donna M. Peehl[a,*], Rajesh Shinghal[a], Larisa Nonn[a], Eugene Seto[a], Aruna V. Krishnan[b], James D. Brooks[a], David Feldman[b]

[a] *Department of Urology, Stanford University School of Medicine, Stanford, CA 94305, USA*
[b] *Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA*

Received 9 March 2004; accepted 9 July 2004

Abstract

1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] exerts anti-proliferative, differentiating and apoptotic effects on prostatic cells. These activities, in addition to epidemiologic findings that link Vitamin D to prostate cancer risk, support the use of 1,25$(OH)_2D_3$ for prevention or therapy of prostate cancer. The molecular mechanisms by which 1,25$(OH)_2D_3$ exerts antitumor effects on prostatic cells are not well-defined. In addition, there is heterogeneity among the responses of various prostate cell lines and primary cultures to 1,25$(OH)_2D_3$ with regard to growth inhibition, differentiation and apoptosis. To understand the basis of these differential responses and to develop a better model of Vitamin D action in the prostate, we performed cDNA microarray analyses of primary cultures of normal and malignant human prostatic epithelial cells, treated with 50 nM of 1,25$(OH)_2D_3$ for 6 and 24 h. CYP24 (25-hydroxyvitamin D_3-24-hydroxylase) was the most highly upregulated gene. Significant and early upregulation of dual specificity phosphatase 10 (DUSP10), validated in five additional primary cultures, points to inhibition of members of the mitogen-activated protein kinase (MAPK) superfamily as a key event mediating activity of 1,25$(OH)_2D_3$ in prostatic epithelial cells. The functions of other regulated genes suggest protection by 1,25$(OH)_2D_3$ from oxidative stress. Overall, these results provide new insights into the molecular basis of antitumor activities of Vitamin D in prostate cells.

Keywords: Vitamin D; Prostate; Vitamin D 24-hydroxylase; Dual specificity phosphatase; Thioredoxin reductase

1. Introduction

Prostate cancer remains the most common non-cutaneous malignancy in U.S. males with over 230,000 cases diagnosed annually. An estimated 28,900 men die each year of the disease, making it the second leading cause of male cancer deaths in the United States [1]. Unfortunately, despite increased screening for prostate cancer, many patients still develop extraprostatic or metastatic disease that is not amenable to conventional surgical or medical therapy. Thus, new emphasis is being placed on identifying methods to prevent this devastating disease. Several promising agents may act by either preventing initiation of malignancy or slowing progression of established disease [2].

The active metabolite of Vitamin D, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], has emerged as a promising preventive or therapeutic agent. In addition to its well-characterized roles in calcium and phosphate regulation and bone metabolism, 1,25$(OH)_2D_3$ promotes cellular differentiation and inhibits proliferation in a variety of tissues by binding to the Vitamin D receptor (VDR) [3]. A large body of work, including our own, suggests that 1,25$(OH)_2D_3$ has potent growth inhibitory and pro-differentiating effects on prostate cells [4–6].

1,25$(OH)_2D_3$ has anti-proliferative effects on prostatic cancer cell lines, including LNCaP and PC-3, as well as on primary prostatic epithelial cell strains [7–9]. A number

* Corresponding author. Tel.: +1 650 725 5531; fax: +1 650 723 4200.
E-mail address: dpeehl@stanford.edu (D.M. Peehl).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.07.003

of mechanisms have been proposed to account for the antiproliferative action of $1,25(OH)_2D_3$, including induction of cell cycle arrest, differentiation, and apoptosis. No single molecular signaling pathway, however, explains the effects of $1,25(OH)_2D_3$ in all cell types. For example, the gene encoding the cyclin-dependent protein kinase inhibitor, p21, may be directly induced by $1,25(OH)_2D_3$ in the leukemia cell line U937 [10], yet p21 levels are unchanged in PC-3 cells despite inhibition of growth of these cells by $1,25(OH)_2D_3$ [11].

The molecular basis of action for $1,25(OH)_2D_3$ in prostatic cells remains poorly understood. The well characterized gene 25-hydroxyvitamin D_3-24-hydroxylase (CYP24), which encodes the enzyme that initiates the catabolism of $1,25(OH)_2D_3$, is induced by $1,25(OH)_2D_3$ in many types of target cells, including prostate cells [3]. Several other $1,25(OH)_2D_3$-regulated genes have also been identified in prostate cancer cell lines. $1,25(OH)_2D_3$ decreases expression of multiple antiapoptotic proteins in LNCaP cells, leading to activation of the mitochondrial pathway for apoptosis [12,13]. $1,25(OH)_2D_3$ also upregulates the expression of androgen receptor and prostate-specific antigen (PSA) in LNCaP cells, but inhibition of this phenomenon by cycloheximide suggests that the effect is indirect and mediated by an unidentified protein [14].

The advent of genome-wide screening methods through the use of complementary DNA (cDNA) microarrays offers a new opportunity to understand regulation of gene expression [15,16]. To better characterize the transcriptional response to $1,25(OH)_2D_3$, we employed cDNA microarrays to assess gene expression profiles of cultured prostatic epithelial cells. Primary cultures derived from normal and malignant prostatic tissues were examined on an array system with 25,000 elements and over 20,000 independent genes and expressed sequence tags (ESTs). Regulation of several candidate molecular targets in additional primary cultures treated with $1,25(OH)_2D_3$ was examined by real-time reverse transcription-polymerase chain reaction (RT-PCR). The functions of the genes regulated by $1,25(OH)_2D_3$ in the primary cultures provoke new ideas about the mechanism of anti-tumor activity of Vitamin D in the prostate.

2. Materials and methods

2.1. Isolation, culture, and Vitamin D treatment of primary prostatic epithelial cells

Tissues were derived from radical prostatectomy specimens that were obtained from men undergoing surgery to treat prostate cancer. The normal cell strain used for microarray analysis (E-PZ-10) was derived from peripheral zone tissue with no histological evidence of cancer in adjacent sections. The cancer cell strain used for microarray studies (E-CA-15) was derived from an adenocarcinoma of Gleason grade 3/3. Two other normal cell strains (E-PZ-1 and E-PZ-2) and three additional cancer cell strains (E-CA-1 from a cancer of Gleason grade 3/4, E-CA-2 from a cancer of grade 3/3, and E-CA-3 from a cancer of grade 4/5) were used to confirm regulation of selected genes by real-time RT-PCR. None of the patients had received prior therapy for prostate cancer. Tissues were processed for culture according to previously described methods [17]. Immunochemical staining for keratin was performed to ensure that the cultures were 100% epithelial. Primary cultures were expanded by serial passage to generate sufficient cells for microarray and RT-PCR analyses (~12–15 population doublings). Semi-confluent cultures were fed fresh medium 2 days prior to treatment. At t_0, 50 nM $1,25(OH)_2D_3$ (Biomol, Plymouth Meeting, PA) or diluent (0.01% ethanol) was added to the medium. Poly A+ RNA (for microarray analysis) or total RNA (for RT-PCR) was isolated at 6 and 24 h, as described below. Each primary culture was treated in duplicate experiments.

2.2. Array production

cDNA microarrays were produced as described previously [18]. cDNA clones were obtained from Research Genetics and the Cancer Genome Anatomy Project in collaboration with the National Cancer Institute, amplified by PCR, and purified as summarized by Ross et al. [19]. The microarrays used in this set of experiments were composed of 24,192 elements (spots) with 23,078 elements containing DNA. The 1114 null spots contained 3× sodium chloride–sodium citrate (SSC) buffer only. Of the clones, 18,433 were sequenced to verify their identity. Over 14,000 elements on the array were uncharacterized ESTs.

2.3. mRNA isolation and array hybridization

Poly A+ RNA was isolated directly from cultured cells using the Invitrogen Fast Track 2.0 method in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). Two micrograms of Poly A+ RNA were reverse transcribed, labeled, purified, and hybridized to the microarray as described previously [18]. Initially, mRNA was annealed to an anchored oligo-DT primer and used to generate cDNA utilizing the Superscript II reverse transcription (Gibco-BRL, Grand Island, NY). For labeling, the reaction was spiked with Cy5-dUTP (treated) or Cy3-dUTP (control) fluorescently-tagged nucleotides. cDNA probes were purified with Microcon-30 (Millipore, Bedford, MA) spin columns and experimental and control probes were mixed. The combined probes were brought up to a volume of 28 ml, placed on the microarray, and hybridized under a glass coverslip in a humidified chamber for approximately 14–16 h at 65 °C. The microarrays were then washed to remove residual probe and scanned using a GenePix 4000 microarray scanner (Axon Instruments Inc., Union City, CA).

2.4. Data analysis

Digitized images of the microarrays were analyzed using Scanalyze software developed by the laboratories of P.

Brown and D. Botstein at Stanford (http://www.microarrays.org/software). Areas with high background or artifact were flagged and excluded from data analysis. The average pixel intensity was determined for each spot on the array. A background intensity was also computed and the net signal was calculated. Data files were submitted to the Stanford Microarray database [20] where gene names are assigned to corresponding spots on the array. Unigene build 150 was used to report gene names and cluster identification numbers. To account for variability in labeling and array background, signal intensities were normalized by scaling the intensities measured in the Cy5 channel.

To ensure high-quality, reproducible data, only those spots with an intensity/background (signal-to-noise) ratio greater than or equal to 1.4 were included in the analysis. Previous experiments have determined that a two-fold or greater change in expression correlates to results that can be reproduced with other methods [21]. Therefore, only genes upregulated or downregulated two-fold or greater are reported. Mean fold-induction or -repression was calculated and reported. If a particular gene was represented by more than one spot on the array, the mean fold-induction or -repression was computed for each array.

2.5. Real-time RT-PCR

Total RNA isolated using an RNeasy kit (Qiagen, Valencia, CA) from cells treated ±50 nM $1,25(OH)_2D_3$ for 6 h was reverse transcribed as described using Thermoscript RT (Invitrogen, Carlsbad, CA). Real-time PCR was performed with the DNA Engine Opticon 2 continuous fluorescence detection system (MJ Research, San Francisco, CA). Reaction contained 100 ng cDNA, 0.3 μM primers and DyNAmo SYBR green qPCR master mix (Finnzymes, Espoo, Finland). Each reaction was done in triplicate to minimize the experimental variations (standard deviation was calculated for each reaction). Each sample was compared to its own treatment control by the calculation $2^{-[cT(treated) - cT(control)]}$. Transcript levels of TATA-box binding protein (TBP) were assayed simultaneously with each of the following seven genes as an internal control to normalize their transcript levels in treated and untreated cells. All primers were supplied by Qiagen and the sequences used were: DUSP10 forward 5′ATCTTGCCCTTCCTGTTCCT3′, DUSP10 reverse 5′ATTGGTGTTTGCCTTTGAC3′; TR1 forward 5′CTTGTGGCCTTTCTGAGGAG3′, TR1 reverse 5′CTGCCAAATGTCAGCTTCA3′; P2RY2 forward 5′TGGCGCTCTACATCTTCTTG3′, P2RY2 reverse 5′TGCTGCAGTAAAGGTTGGTG3′; SURV forward 5′CCACCGCATGTCTACATTCA3′, SURV reverse 5′GCACTTTCTTCGCAGTTTCC3′; BMP-6 forward 5′TGAGCTTTGTGAACCTGGTG3′, BMP-6 reverse 5′GAGGCCCCATGTTATGCTGT3′; CTGF forward 5′TTCCAGAGCAGCTGCAAGTA3′, CTGF reverse 5′ATCCCACAGGTCTTGGAACA3′; MET1H forward 5′GCTCCTGCAAGTGCAAAAAG3′, MET1H reverse 5′CAGCAGCTGCACTTCTCTGA3′; TBP forward 5′TGCTGAGAAGAGTGTGCTGGAG3′ and TBP reverse 5′TCTGAATAGGCTGTGGGGTC3′.

3. Results

3.1. Normal prostatic epithelial cells: genes upregulated in response to 1,25(OH)$_2$D$_3$

Initial experiments focused on the response of normal prostatic epithelial cells to $1,25(OH)_2D_3$. Semi-confluent cells (E-PZ-10) in standard serum-free growth medium were treated with or without 50 nM $1,25(OH)_2D_3$ for 6 and 24 h in duplicate experiments and gene expression profiles were generated on separate arrays. This dose of $1,25(OH)_2D_3$ was selected because it has been shown to significantly inhibit the growth of these cells [22].

A list of genes upregulated by $1,25(OH)_2D_3$ in normal cells is presented in Table 1. Genes were classified as early (6 h only), late (24 h only), or early and sustained (6 and 24 h) based on the fold-increase at each time point. Only those genes that exhibited two-fold or more increased expression in both experiments are reported to ensure the fidelity of the data. The fold-increase at both time points for those genes that were induced two-fold at only 6 or 24 h is presented to demonstrate a pattern of expression. Only named genes are reported here.

For the 6-h treatment, 48 genes that met selection criteria were identified. Twenty-nine of these were named genes and 19 were ESTs. Duplicate spots were present on the array for three of these genes. At the 24-h time point, 38 genes were increased two-fold or more. Twenty-eight were named genes and 10 were ESTs.

The majority of these genes were increased at least two-fold and remained upregulated at the 24-h time point. A robust induction of nearly 80-fold was seen for the Vitamin D 24-hydroxylase gene (CYP24). A strong, early induction of several metallothionein genes was observed as well.

3.2. Normal prostatic epithelial cells: genes downregulated in response to 1,25(OH)$_2$D$_3$

A list of genes downregulated two-fold or more in normal cells by treatment with 50 nM of $1,25(OH)_2D_3$ is presented in Table 2. In contrast to the rather large number of genes upregulated by $1,25(OH)_2D_3$, relatively few genes were downregulated at the time points assayed. At 6 h, 24 genes were downregulated a minimum of two-fold. Six of these were named genes and 18 were ESTs. At 24 h, 39 genes were repressed at least two-fold or more (3 named genes and 36 ESTs). Only six genes, all ESTs, were repressed two-fold or more at both time points on both microarrays.

Table 1
Genes upregulated in normal prostatic epithelial cells by $1,25(OH)_2D_3$

Unigene accession number	Gene name	Fold-change	
		6 h	24 h
Early response only			
Hs.177534	Dual specificity phosphatase 10	3.14	1.95
Hs.13046	Thioredoxin reductase 1	3.09	1.78
Hs.23731	Discs, large (Drosophila) homolog 4	2.84	1.50
Hs.788	A kinase (PRKA) anchor protein (gravin) 12	2.76	1.55
Hs.2030	Thrombomodulin	2.43	1.66
Hs.169300	Transforming growth factor, beta 2	2.37	1.68
Hs.166071	Cyclin-dependent kinase 5	2.36	1.50
Hs.72912	Cytochrome P450, subfamily I (aromatic compound-inducible), polypeptide 1	2.30	-1.03
Hs.74111	RNA-binding protein (autoantigenic)	2.07	1.22
Early and sustained response			
Hs.89663	Cytochrome P450, subfamily XXIV (Vitamin D 24-hydroxylase)	79.15	82.57
Hs.2667	Metallothionein 1H	12.66	5.30
Hs.94360	Metallothionein 1L	12.02	2.76
Hs.66	Interleukin 1 receptor-like 1	9.41	4.13
Hs.334409	Metallothionein 1G	9.18	6.67
Hs.8765	RNA helicase-related protein	8.96	3.83
Hs.144567	Alanine-glyoxylate aminotransferase (oxalosis I; hyperoxaluria I; glycolicaciduria; serine-pyruvate aminotransferase)	6.61	2.65
Hs.74170	Metallothionein 1E (functional)	6.15	2.44
Hs.285671	Bone morphogenetic protein 6	3.92	3.02
Hs.80658	Uncoupling protein 2 (mitochondrial, proton carrier)	3.89	2.59
Hs.8230	Homo sapiens a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1 (ADAMTS1), mRNA	3.53	2.81
Hs.76252	Endothelin receptor type A	3.17	2.40
Hs.1578	Apoptosis inhibitor 4 (survivin)	3.12	2.87
Hs.339	Purinergic receptor P2Y, G-protein coupled, 2	2.63	2.28
Hs.135150	Lung type-I cell membrane-associated glycoprotein	2.56	2.42
Hs.201967	Aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	2.52	2.17
Hs.51120	Cathelicidin antimicrobial peptide	2.49	4.95
Hs.155097	Carbonic anhydrase II	2.43	3.23
Hs.25155	Neuroepithelial cell transforming gene NET1	2.33	2.02
Hs.183583	Serine proteinase inhibitor, clade B, member 1	2.22	2.20
Late response only			
Hs.79022	GTP-binding protein overexpressed in skeletal muscle	1.94	3.79
Hs.76884	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	1.66	3.23
Hs.239600	Calmodulin-like 3	1.63	3.00
Hs.332053	Serum amyloid A1	1.58	2.84
Hs.82085	Serine protease inhibitor, SERPINE 1	1.39	2.45
Hs.77348	Hydroxyprostaglandin dehydrogenase 15-(NAD)	1.16	2.34
Hs.180919	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	1.03	2.32
Hs.146688	Microsomal glutathione *S*-transferase 1-like 1/prostaglandin E synthase	1.65	2.16
Hs.198951	Jun B proto-oncogene	1.64	2.10

Table 2
Genes downregulated in normal prostatic epithelial cells by $1,25(OH)_2D_3$

Unigene accession number	Gene name	Mean fold downregulated
Early response (6 h)		
Hs.1074	Surfactant, pulmonary-associated protein C	−2.86
Hs.104624	Aquaporin 9	−2.63
Hs.283006	Phospholipase C, beta 4	−2.57
Hs.85112	Insulin-like growth factor 1 (somatomedin C)	−2.22
Hs.17287	Potassium inwardly-rectifying channel, subfamily J, member 15	−2.04
Hs.75613	CD36 antigen (collagen type I receptor, thrombospondin receptor)	−2.00
Late response (24 h)		
Hs.227011	G-substrate	−3.03
Hs.26530	Serum deprivation response (phosphatidylserine-binding protein) (SDPR)	−2.63
Hs.181581	Glutamate receptor, ionotropic, kainate 1	−2.23

Table 3
Genes upregulated in prostate cancer cells by $1,25(OH)_2D_3$

Unigene accession number	Gene name	Fold-change	
		6 h	24 h
Early response only			
Hs.177534	Dual specificity phosphatase 10	3.42	0.51
Hs.339	Purinergic receptor P2Y, G-protein coupled, 2	2.93	0.92
Hs.13046	Thioredoxin reductase 1	2.80	1.57
Hs.788	A kinase (PRKA) anchor protein (gravin) 12	2.49	0.72
Hs.87435	Homo sapiens mRNA for neuroblastoma, complete cds	2.35	1.52
Hs.72912	Cytochrome P450, subfamily I (aromatic compound-inducible), polypeptide 1	2.22	0.17
Hs.119122	Ribosomal protein L13a	2.07	1.06
Hs.155017	Nuclear receptor interacting protein 1	2.03	1.51
Hs.6557	Zinc finger protein 161	2.02	1.73
Early and sustained response			
Hs.89663	Cytochrome P450, subfamily XXIV (Vitamin D 24-hydroxylase)	78.03	46.12
Hs.66	Interleukin 1 receptor-like 1	4.58	3.22
Hs.6101	Bone morphogenetic protein 6	2.81	3.16
Hs.51120	Cathelicidin antimicrobial peptide	2.50	4.33
Hs.8230	Disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1 (ADAMTS1)	2.13	2.08
Hs.155097	Carbonic anhydrase II	2.14	4.1
Hs.183583	Protease inhibitor 2 (anti-elastase), monocyte/neutrophil	2.10	2.54
Late response only			
Hs.115770	Tumor necrosis factor (ligand) superfamily, member 11	0.99	4.26
Hs.226213	Cytochrome P450, 51 (lanosterol 14-alpha-demethylase)	1.07	2.62
Hs.196416	Dihydrolipoamide *S*-succinyltransferase (E2 component of 2-oxo-glutarate complex)	0.98	2.24
Hs.153863	MAD (mothers against decapentaplegic, Drosophila) homolog 6	1.02	2.10
Hs.177781	Superoxide dismutase 2, mitochondrial	1.01	2.04

3.3. Prostate cancer-derived epithelial cells: genes upregulated by $1,25(OH)_2D_3$

Gene expression profiles were also generated for an epithelial cell strain derived from a prostatic adenocarcinoma of Gleason grade 3/3 (E-CA-15). As in the case of the normal cell strain, semi-confluent cultures of the cancer cells in standard medium were treated with or without 50 nM of $1,25(OH)_2D_3$ and mRNA was isolated at 6 and 24 h. As for the normal cells, this dose of $1,25(OH)_2D_3$ inhibited the growth of the cancer cells. A list of upregulated genes using selection criteria identical to those used for the normal epithelial cell strain is presented in Table 3.

At 6 h, a total of 27 genes was upregulated two-fold or more (16 named genes and 11 ESTs). Only the named genes are presented in Table 3. At 24 h, 37 upregulated genes were identified (12 named genes and 25 ESTs). The number of genes upregulated was similar to the number observed with the normal strain treated with $1,25(OH)_2D_3$.

A number of similarities were observed between the gene expression profiles generated from normal and cancer-derived epithelial cell strains after treatment with $1,25(OH)_2D_3$. At 6 h, dual specificity phosphatase 10 (DUSP10), thioredoxin reductase 1 (TR1), purinergic receptor (P2RY2), A kinase (PRKA) anchor protein (gravin) 12, and a cytochrome P450 enzyme (CYP1A1) were noted in both cell strains in the early response group.

At both 6 and 24 h, upregulation of Vitamin D 24-hydroxylase was noted again in the cancer-derived epithelial strain, confirming a biologic response to the treatment. Genes in common between normal and cancer-derived cells that showed an early and sustained response included interleukin 1 receptor-like 1, bone morphogenetic protein 6 (BMP-6), carbonic anhydrase II, and protease inhibitor 2 (anti-elastase). There was a notable absence of the metallothionein genes, RNA helicase-related protein, and alanine-glyoxylate aminotransferase in the list of genes upregulated in the cancer compared to the normal cell strain. Moreover, the expression of the metallothionein genes was appreciably down-regulated in the cancer cell strain (Table 4, see below).

3.4. Prostate cancer-derived epithelial cell strain: genes downregulated by $1,25(OH)_2D_3$

Finally, those genes downregulated two-fold or more at 6 and 24 h by $1,25(OH)_2D_3$ in cancer cells are presented in Table 4. In contrast to the normal prostate epithelial cell strain, a large number of genes was downregulated at both time points. After 6 h of treatment, 14 genes were downregulated two-fold or more (6 named genes and 8 ESTs). Of the early response-only genes, 24-h data was not available for three of the four genes because the spot intensity was too low to allow interpretation.

After 24 h of exposure to $1,25(OH)_2D_3$, 139 genes were downregulated two-fold or more (68 named genes and 71 ESTs). A subset of these genes that were most potently repressed is listed in Table 4. Only two genes, fibronectin 1 and

Table 4
Genes downregulated in prostate cancer cells by $1,25(OH)_2D_3$

Unigene accession number	Gene name	Fold-change	
		6 h	24 h
Early response only			
Hs.2391	Apical protein, *Xenopus laevis*-like	−2.51	−1.64
Hs.57922	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	−2.45	Flag
Hs.17287	Potassium inwardly-rectifying channel, subfamily J, member 15	−2.30	Flag
Hs.8265	Transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	−2.21	Flag
Early and sustained response			
Hs.287820	Fibronectin 1	−2.47	−7.68
Hs.75511	Connective tissue growth factor	−2.02	−2.91
Late response only			
Hs.283006	Phospholipase C, beta 4	−1.09	−7.66
Hs.418	Fibroblast activation protein, alpha	−1.41	−7.08
Hs.72912	Cytochrome P450, subfamily I (aromatic compound-inducible), polypeptide 1	−0.45	−5.86
Hs.118787	Transforming growth factor, beta-induced, 68 kD	−1.47	−5.64
Hs.75777	Transgelin	−1.96	−4.83
Hs.83337	Latent transforming growth factor beta binding protein 2	−1.06	−4.60
Hs.276916	Nuclear receptor subfamily 1, group D, member 1	−1.32	−4.58
Hs.326035	Early growth response 1	−1.13	−4.17
Hs.173451	Metallothionein 1G	−1.24	−4.11
Hs.2667	Metallothionein 1H	−1.32	−4.08
Hs.144567	Alanine-glyoxylate aminotransferase	−1.21	−3.98
Hs.8765	RNA helicase-related protein	−1.14	−3.92
Hs.184641	Fatty acid desaturase 2	−0.99	−3.85
Hs.76669	Nicotinamide *N*-methyltransferase	−1.38	−3.63
Hs.75617	Collagen, type IV, alpha 2	−1.25	−3.58
Hs.87409	Thrombospondin 1	−1.59	−3.54
Hs.10082	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	−1.08	−3.52
Hs.278503	Regulated in glioma	−0.91	−3.45
Hs.70327	Cysteine-rich protein 2	−1.07	−3.45
Hs.173894	Colony stimulating factor 1 (macrophage)	−1.02	−3.44
Hs.77274	Plasminogen activator, urokinase	−1.04	−3.44
Hs.94360	Metallothionein 1 L	−1.26	−3.20
Hs.119129	Collagen, type IV, alpha 1	−1.57	−3.14
Hs.301865	Dopachrome tautomerase	−1.31	−3.03
Hs.334562	Cell division cycle 2, G1 to S and G2 to M	−0.90	−2.96

connective tissue growth factor (CTGF), were observed to be repressed two-fold or more at both 6 and 24 h.

3.5. Validation of regulation of selected genes by real-time RT-PCR

Insufficient RNA remained after the microarray analyses to validate regulation of genes by $1,25(OH)_2D_3$ in the same samples by real-time RT-PCR. Therefore, two additional normal cell strains (E-PZ-1 and E-PZ-2) and three additional cell strains from cancer (E-CA-1, E-CA-2 and E-CA-3) were treated with or without 50 nM of $1,25(OH)_2D_3$ and RNA was isolated at 6 h. Real-time PCR studies confirmed the microarray data in the case of some of the key genes that demonstrated regulation by $1,25(OH)_2D_3$ as described below.

Four genes that were upregulated in both normal and cancer cells at 6 h in the microarray analyses – DUSP10, TR1, P2RY2 and BMP-6 – were measured by real-time RT-PCR (Fig. 1). DUSP10 and TR1 were significantly upregulated by $1,25(OH)_2D_3$ in all of the samples, with a mean of ∼3-fold increase, which compared favorably with the microarray results. While P2RY2 was significantly upregulated by $1,25(OH)_2D_3$ in both of the normal cell strains, this gene was significantly induced in only one of three cancer cell strains. The mean fold-induction in the normal cells was ∼2.6, which was comparable to the fold-induction in the microarray analysis. BMP-6 was upregulated in one of two normal cell strains, and in one of two cancer strains.

We also evaluated several additional genes that had shown differential regulation by $1,25(OH)_2D_3$ in normal versus cancer cells in the microarrays. These studies revealed some differences between gene expression data obtained by real-time PCR assays and microarray experiments. For example, survivin (SURV) showed an early and sustained upregulation in normal but not cancer cells in the arrays, but SURV mRNA levels were unchanged by $1,25(OH)_2D_3$ in either normal or cancer cells by real-time RT-PCR analysis (Fig. 1). CTGF was preferentially downregulated by $1,25(OH)_2D_3$ in cancer cells in the arrays, but was unchanged in either cancer or normal cells by real-time RT-PCR analysis (Fig. 1). Metallothionein

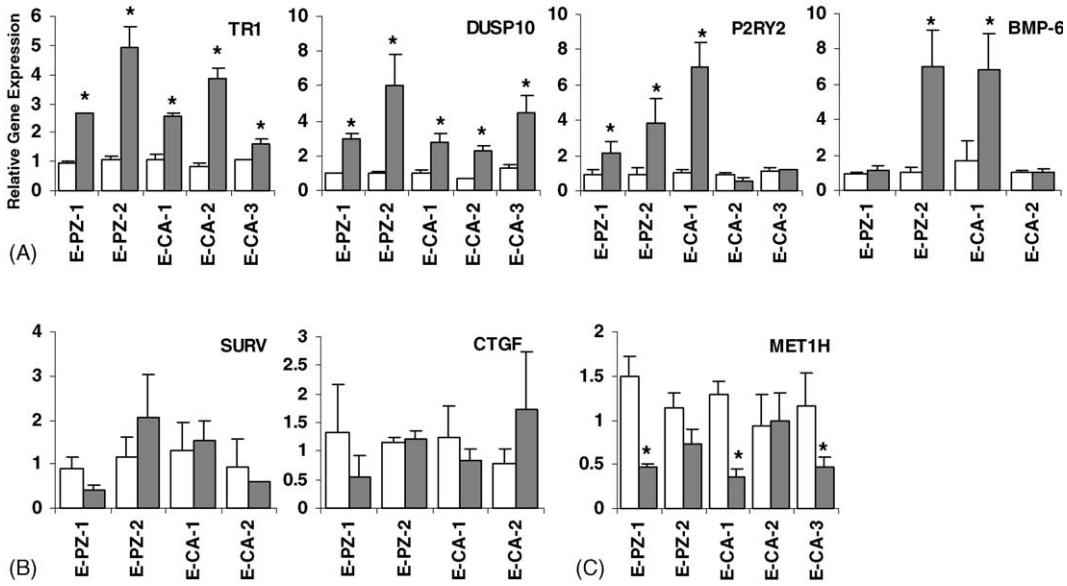

Fig. 1. Analysis of gene expression by real-time RT-PCR 6 h after treatment with diluent (open bars) or 50 nM 1,25D (shaded bars). Genes that were shown to be: (A) up-regulated, (B) down-regulated and (C) differentially regulated by micro-array analysis. Thioredoxin reductase 1 (TR1), dual-specificity phosphatase 10 (DUSP10), purinergic 2 receptor Y2 (P2RY2), bone morphogenetic protein 6 (BMP-6), survivin (SURV), connective tissue growth factor (CTGF) and methallothionein 1H (MET1H). Expression is relative to diluent treatment for each cell strain and normalized to the expression of TATA-box binding protein (TBP). Error bars are standard deviation of triplicate reactions, $*p < 0.05$ compared to diluent.

1H (MET1H) had shown a large increase (13-fold) by 6 h in normal cells in the array but downregulation in cancer cells. By real-time RT-PCR analysis, MET1H was significantly downregulated in two of three cancer strains, but also in one of two normal cell strains, and there was no up-regulation in the other normal cell strain (Fig. 1). Overall, these results suggest that the differential gene regulation by $1,25(OH)_2D_3$ in the arrays may be due to inter-cell strain variability or other factors unrelated to normal versus malignant phenotypes.

4. Discussion

Microarray technology has proven to be a powerful new tool to identify changes in gene expression caused by factors that regulate cell growth. One emerging tenet is that there is a great deal of cell-type specificity in gene expression profiles generated by a given factor. Furthermore, the genetic background upon which the factor works has a significant impact on the final outcome. Even related cell types can show very different responses to a specific factor. Given that established cell lines have many genetic abnormalities, it is particularly worthwhile to use primary cultures to delineate responses to Vitamin D. The normal cells in this study were derived from the peripheral zone of the adult human prostate, the region of the prostate with the highest incidence of cancer [23]. The phenotype of these cells in vitro is most like that of "transit amplifying cells" and markers of both basal and secretory cells are expressed [24]. In order to mimic the low proliferation rate of such cells in tissues, the cultured cells were fed fresh medium two days prior to treatment with Vitamin D. At this time, the mitogen-induced peak of proliferation has passed and cells are returning to quiescence. The cell from which prostate cancer originates is unknown, but it is likely to be a transit amplifying cell or perhaps a stem cell. In any event, effects of Vitamin D on normal cells are relevant to prevention of prostate cancer. Similarly, the cancer cell strains chosen for this analysis were derived from clinically localized tumors, representative of early stage disease. At this time, the majority of men are diagnosed with early stage prostate cancer, and therefore responses of these cells are relevant to chemotherapeutic activity of Vitamin D, currently being tested in clinical trials.

Gene expression profiles generated by a given factor are tightly linked to the biological outcome of treatment with the factor. For example, in a companion study we reported the gene expression profile of LNCaP cells, an established prostate cancer cell line, after exposure to 50 nM of $1,25(OH)_2D_3$ for 6 and 24 h [25]. We found only one gene regulated in common between the LNCaP cells and the primary cell cultures, which was hydroxyprostaglandin dehydrogenase 15-(NAD). This result perhaps reflects the differences in biological activity of $1,25(OH)_2D_3$ in the established cell line versus the primary cultures. $1,25(OH)_2D_3$ causes growth inhibition and apoptosis in LNCaP cells [12,13], whereas it causes only growth inhibition in primary cultures [9]. Suppression of growth of LNCaP cells by $1,25(OH)_2D_3$ is reversible [26] whereas it is irreversible in primary cultures even after as little as 2 hr of exposure [9].

$1,25(OH)_2D_3$ upregulates expression of androgen receptor and PSA in LNCaP cells [14,27,28], whereas primary cultures do not express androgen receptors or PSA and these genes are not induced by $1,25(OH)_2D_3$.

The induction of Vitamin D 24-hydroxylase was of the highest magnitude among all genes upregulated by $1,25(OH)_2D_3$ in both normal and cancer-derived primary cultures. The promoter of this gene contains Vitamin D response elements (VDREs) and its regulation by Vitamin D is very important in maintaining Vitamin D homeostasis in the body as the enzyme it encodes converts $1,25(OH)_2D_3$ to biologically less active metabolites [3]. It is interesting to note that cells resistant to growth inhibition by $1,25(OH)_2D_3$ still often upregulate Vitamin D 24-hydroxylase when treated with $1,25(OH)_2D_3$ [29–31]. Yet this gene is expressed at barely detectable levels in LNCaP cells, and in our companion microarray study [25] we did not detect any increases in 24-hydroxylase in LNCaP cells even after exposure to 50 nM $1,25(OH)_2D_3$, confirming our previously reported results using Northern blot analyses [8].

What is the significance of other genes regulated by $1,25(OH)_2D_3$ in primary cultures of prostatic epithelial cells? Of the early response genes, DUSP10 was the most highly upregulated in both normal and cancer-derived cultures in the array analyses. Confirmation of similar upregulation in two additional normal cultures and three additional cancer cultures by real-time RT-PCR unequivocally demonstrates that DUSP10 is involved in molecular signaling by $1,25(OH)_2D_3$ in prostatic epithelial cells. Interestingly, DUSP10 was also significantly upregulated by $1,25(OH)_2D_3$ in human colon cancer cells [32]. DUSP10 belongs to the non-receptor class of the protein tyrosine phosphatase family [33]. This phosphatase inactivates mitogen-activated protein kinases (MAPKs), with specificity for p38 and jun N-terminal kinase (JNK) [34]. In this regard, it is noteworthy that repression of the stress-activated protein kinases (SAPKs) p38 and JNK has been implicated in Vitamin D's activities in several other types of cells. For example, several studies showed that Vitamin D inhibited the activation of p38 and JNK by ultraviolet irradiation and other stresses in keratinocytes, suggesting that inhibition of SAPKs might account for the protective effects of $1,25(OH)_2D_3$ on epidermal cells during exposure to sunlight [35,36]. Another study showed that inhibition of the p38 MAPK pathway potentiated $1,25(OH)_2D_3$-induced monocytic differentiation of human leukemia HL60 cells, again suggesting a role for inhibition of SAPKs in Vitamin D activity [37]. Based on our results, the inhibition of SAPKs by Vitamin D may be mediated by the induction of DUSP10 or other phosphatases with similar specificity.

TR1, which was induced by $1,25(OH)_2D_3$ in 100% of our primary cultures, is involved in maintaining redox balance. TR1 was also upregulated in two breast cancer cell lines by $1,25(OH)_2D_3$ [38]. TR1 is a selenoflavoenzyme primarily known for its role in the thioredoxin system as a reducing enzyme for thioredoxin (Trx). Many aggressive cancers have been shown to overexpress TR1 and Trx [39]. Whereas overexpression of Trx alone or with TR1 causes increased cellular proliferation, increased levels of TR1 alone do not affect cell growth [40]. Because TR1 is a selenium-containing enzyme, it has been problematic to study experimentally and little is known about activities of TR1 outside the thioredoxin system. However, TR1 has also been shown to have antioxidant properties by directly reducing other substrates such as ascorbate, lipoic acid, lipid hydroperoxides and vitamin K3, but the role of TR1 in physiological regulation of these substrates is not clear [40].

Induction of TR1 is consistent with a protective effect of Vitamin D against oxidative damage. This finding is relevant to an area of controversy in the Vitamin D field regarding whether $1,25(OH)_2D_3$ acts as an oxidant or antioxidant. Koren et al. [41] found that the effects of $1,25(OH)_2D_3$ on breast cancer cells were consistent with oxidant activity. Vitamin D increased reactive oxygen species (ROS), and this was proposed to be the basis of Vitamin D's ability to potentiate the antiproliferative activity of other drugs that increase ROS. Vitamin D also reduced Cu/Zn superoxide dismutase (SOD) RNA in additional studies [42]. Overall, the antioxidant capacity of the cells was decreased, increasing the effects of ROS-generating drugs.

Garcion et al. [43] offered a somewhat different interpretation of Vitamin D's activities. They suggested that Vitamin D exerts a sublethal oxidative stress, which stimulates cells to upregulate detoxification mechanisms to protect themselves from the challenge. In their studies, they found that $1,25(OH)_2D_3$ increased the activity of γ-glutamyl transpeptidase in neural cells, which led to an increase in intracellular glutathione (GSH). In this way, Vitamin D participated in antioxidant machinery by increasing GSH that protected cells from further oxidative challenge. Our results suggest that Vitamin D protects prostate cells from oxidative damage by upregulating TR1.

BMP-6 is another interesting gene that showed an early and sustained induction by $1,25(OH)_2D_3$ in both normal and cancer-derived cells in the arrays, and in one of two normal and one of two cancer cultures by real-time RT-PCR. A member of the transforming growth factor (TGF)-β superfamily, BMP-6 is particularly prominent in metastatic cells compared to primary prostatic adenocarcinomas and BMP-6 has been implicated in the formation of osteoblastic metastases by prostate cancer [44]. However, BMP-6 is also expressed by basal cells of the glandular epithelium of normal prostatic tissues [45], so its expression in both normal and cancer-derived prostatic cell cultures would not be unanticipated. In published gene expression profiles of other types of cells treated with $1,25(OH)_2D_3$, one or another member of the BMP or TGFβ families often appears, suggesting a widespread role for this superfamily in Vitamin D action.

We were unable to validate by real-time RT-PCR some of the genes that appeared to be differentially regulated by $1,25(OH)_2D_3$ between normal and cancer cells in the arrays. Expression of neither SURV or CTGF was significantly changed by $1,25(OH)_2D_3$ in two of two normal and two of two

cancer cell strains in the RT-PCR assays. MET1H, which was downregulated in cancer cells by $1,25(OH)_2D_3$ in the arrays, was downregulated in two of three cancer cell strains in the RT-PCR analysis but was also downregulated in 1/2 normal cell strains. This result with normal cells was not consistent with the arrays, where MET1H had been appreciably upregulated in normal cells. There findings may reflect inter-cell strain heterogeneity, or lack of validation may be a consequence of uncontrolled culture variables. While we tried to keep cell density consistent in each experiment and always changed medium two days prior to treatment with Vitamin D or vehicle, it is not as easy to control culture variables with primary cultures as it is with established cell lines. The genes that we could not validate by RT-PCR may be particularly sensitive to environmental conditions. Additional studies will be required to determine whether there are any reproducible cancer-specific differences in molecular targets of $1,25(OH)_2D_3$ that correlate with different functional responses.

Our results are also noteworthy for the absence of regulation of certain genes by $1,25(OH)_2D_3$. More than 50 genes have been described as sensitive to Vitamin D, including at least 26 genes containing promoters in which VDREs have been identified [46–48]. Osteocalcin, for example, was not regulated in primary cultures of prostatic cells, despite the fact that the promoter of this gene has a VDRE. In contrast, the prostate cancer cell line PC-3 expresses osteocalcin, and an osteocalcin promoter-luciferase construct exhibited strong Vitamin D-induced activity in these cells [49]. The expression of the cell cycle inhibitor p21 also was not increased by $1,25(OH)_2D_3$ in our cell strains. We showed that p21 mRNA expression was increased in LNCaP cells in response to Vitamin D, but the effect was possibly indirect and mediated by insulin-like growth factor binding protein-3 (IGFBP-3) [50]. Reports regarding the role of p21 in mediating signaling by Vitamin D are variable. Although the promoter of p21 has a VDRE [10], transcriptional regulation of p21 by Vitamin D is not consistently seen in different types of cells [51].

The expression of the IGFBP-3 gene showed the highest fold-increase after $1,25(OH)_2D_3$ treatment in our companion study with LNCaP cells (33-fold induction at 24 h) [25], yet this gene was not regulated by $1,25(OH)_2D_3$ in our analysis of primary cultures. IGFBP-3 has been implicated as a key mediator of Vitamin D activity in LNCaP cells [50]. We did not see regulation of IGFBP-3 and in fact we have never observed expression of IGFBP-3 in primary cultures of prostatic epithelial cells. Clearly, IGFBP-3 is not mandatory for growth inhibition of prostatic epithelial cells and may instead be linked to apoptosis induced by Vitamin D [52]. Vitamin D does not induce apoptosis in primary cultures, and the lack of regulation of IGFBP-3 as well as of bcl-2 by Vitamin D is consistent with this response. In contrast, Vitamin D alters the ratio of expression of bcl-2/bax in cells which undergo apoptosis in response to Vitamin D [12,13,53].

Results from cDNA microarray analyses of squamous skin carcinoma cell lines [31], human colon cancer cell lines [32], T-cells [54] and mouse mammary cell lines [55] treated with $1,25(OH)_2D_3$ were recently published. In addition to the common induction of Vitamin D 24-hydroxylase in many types of cells, and the aforementioned DUSP10 in colon cancer cells and TR1 in human breast cancer cells, there were a few other Vitamin D-regulated genes in common between primary cultures of prostatic epithelial cells and other cell types. Upregulation of members of the jun family by $1,25(OH)_2D_3$ was reported in colon and mammary cell lines [32,55], and we observed induction of junB in our prostatic cells. Therefore, elevated levels of jun may contribute to antiproliferative activity of Vitamin D in many types of cells. Other genes regulated in both prostate and colon cells were gravin and carbonic anhydrase. Gravin is a kinase anchoring protein that coordinates the localization of protein kinase C (PKC) and PKA [56]. Several studies have shown that $1,25(OH)_2D_3$ acts on cells through membrane-mediated mechanisms, resulting in rapid changes that include an increase in PKC-specific activity [57]. Early upregulation of gravin by $1,25(OH)_2D_3$ is perhaps related to the recently reported $1,25(OH)_2D_3$-regulated packaging of PKC in chondrocytes [58]. Although TR1 was not regulated by $1,25(OH)_2D_3$ in colon cells as in prostatic and human breast cancer cells, the induction by $1,25(OH)_2D_3$ of glutathione peroxidase in colonic cells may have similar effects on cellular redox status [32]. Overall, some common pathways of molecular action of Vitamin D in diverse cell types are emerging.

In summary, our results provide a number of new insights into the mechanism of action of Vitamin D in primary cultures of prostatic epithelial cells. Our study revealed several novel target genes and detailed analyses of the role of these genes in mediating anti-tumor effects of Vitamin D may lead to better approaches to prevent or cure prostate cancer with $1,25(OH)_2D_3$ or analogs.

Acknowledgements

This work was supported in part by NIH Training Grant 5-T32-DK07217 (L.N.), the Doris Duke Foundation (T98064) (R.J., J.D.B.), NIH DK42482 (A.V.K., D.F.) and the Cancer Research Fund (D.M.P., E.S.), under Interagency Agreement #97-12013 (University of California, Davis contract #98-00924V) with the Department of Health Services, Cancer Research Section. Mention of trade name, proprietary product or specific equipment does not constitute a guaranty or warranty by the Department of Health Services, nor does it imply approval to the exclusion of other products. The views expressed herein represent those of the authors and do not necessarily represent the position of the State of California, Department of Health Services.

References

[1] R.T. Greenlee, M.B. Hill-Harmon, T. Murray, M. Thun, Cancer statistics, 2001, CA, Cancer J. Clin. 51 (2001) 15–36.

[2] R. Lieberman, C. Bermejo, H. Akaza, P. Greenwald, W. Fair, I. Thompson, Progress in prostate cancer chemoprevention: modulators of promotion and progression, Urology 58 (2001) 835–842.
[3] D. Feldman, P.J. Malloy, C. Gross, Vitamin D: biology, action and clinical implications, in: R. Marcus, D. Feldman, J. Kelsey (Eds.), Osteoporosis, vol. 1, Academic Press, San Diego, 2001 (Chapter 9, pp. 257–304).
[4] A.V. Krishnan, D.M. Peehl, D. Feldman, Inhibition of prostate cancer growth by Vitamin D: regulation of target gene expression, J. Cell Biochem. (2003) 88.
[5] G. Miller, Vitamin D and prostate cancer: biologic interactions and clinical potentials, Cancer Met. Rev. 17 (1999) 353–360.
[6] S.E. Blutt, N.L. Weigel, Vitamin D and prostate cancer, Proc. Soc. Exp. Biol. Med. 221 (1999) 89–98.
[7] G.J. Miller, G.E. Stapleton, T.E. Hedlund, K.A. Moffat, 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 (1995) 997–1003.
[8] R.J. Skowronski, D.M. Peehl, D. Feldman, Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D3 receptors and actions in human prostate cancer cell lines 132 (1993) 1952–1960.
[9] D.M. Peehl, R.J. Skowronski, G.K. Leung, S.T. Wong, T.A. Stamey, D. Feldman, Antiproliferative effects of 1,25-dihydroxyvitamin D3 on primary cultures of human prostatic cells, Cancer Res. 54 (1994) 805–810.
[10] M. Liu, M.H. Lee, M. Cohen, M. Bommakanti, L.P. Freedman, Transcriptional activation of the Cdk inhibitor p21 by Vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937, Genes Dev. 10 (1996) 142–153.
[11] S.H. Zhuang, K.L. Burnstein, 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 (1998) 1197–1207.
[12] S.E. Blutt, T.J. McDonnell, T.C. Polek, N.L. Weigel, Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2, Endocrinology 141 (2000) 10–17.
[13] M. Guzey, S. Kitada, J.C. Reed, Apoptosis induction by 1alpha,25-dihydroxyvitamin D3 in prostate cancer, Mol. Cancer Ther. 1 (2002) 667–677.
[14] X.Y. Zhao, L.H. Ly, D.M. Peehl, D. Feldman, Induction of androgen receptor by 1alpha,25-dihydroxyvitamin D3 and 9-*cis* retinoic acid in LNCaP human prostate cancer cells, Endocrinology 140 (1999) 1205–1212.
[15] M. Schena, D. Shalon, R.W. Davis, P.O. Brown, Quantitative monitoring of gene expression patterns with a complementary DNA microarray, Science 270 (1995) 467–470.
[16] D. Shalon, S.J. Smith, P.O. Brown, A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization, Genome Res. 6 (1996) 639–645.
[17] D.M. Peehl, Human prostatic epithelial cells, in: R.I. Freshney, M.G. Freshney (Eds.), Culture of Epithelial Cells, Wiley-Liss Inc., New York, NY, 2002, pp. 159–180.
[18] M.B. Eisen, P.O. Brown, DNA arrays for analysis of gene expression, Methods Enzymol. 303 (1999) 179–205.
[19] D.T. Ross, U. Scherf, M.B. Eisen, C.M. Perou, C. Rees, P. Spellman, V. Iyer, S.S. Jeffrey, M. Van de Rijn, M. Waltham, A. Pergamenschikov, J.C. Lee, D. Lashkari, D. Shalon, T.G. Myers, J.N. Weinstein, D. Botstein, P.O. Brown, Systematic variation in gene expression patterns in human cancer cell lines, Nat. Genet. 24 (2000) 227–235.
[20] G. Sherlock, T. Hernandez-Boussard, A. Kasarskis, G. Binkley, J.C. Matese, S.S. Dwight, M. Kaloper, S. Weng, H. Jin, C.A. Ball, M.B. Eisen, P.T. Spellman, P.O. Brown, D. Botstein, J.M. Cherry, The Stanford Microarray Database, Nucleic Acids Res. 29 (2001) 152–155.
[21] V.R. Iyer, M.B. Eisen, D.T. Ross, G. Schuler, T. Moore, J.C. Lee, J.M. Trent, L.M. Staudt, J. Hudson Jr., M.S. Boguski, D. Lashkari, D. Shalon, D. Botstein, P.O. Brown, The transcriptional program in the response of human fibroblasts to serum, Science 283 (1999) 83–87.
[22] J.Y. Hsu, D. Feldman, J.E. McNeal, D.M. Peehl, Reduced 1alpha-hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D3-induced growth inhibition, Cancer Res. 61 (2001) 2852–2856.
[23] J.E. McNeal, Prostate, in: S.S. Sternberg (Ed.), Histology for Pathologists, Lippinocott-Raven, Philadelphia, 1997, pp. 997–1017.
[24] A.R. Uzgare, Y. Xu, J.T. Isaacs, In vitro culturing and characteristics of transit amplifying epithelial cells from human prostate tissue, J. Cell Biochem. 91 (2004) 196–205.
[25] A.V. Krishnan, S.R.N. Raghavachari, J.D. Brooks, D.M. Peehl, D. Feldman, Analysis of Vitamin D-regulated gene expression in LNCaP human prostate cancer cells using cDNA microarrays, Prostate. (2004) 243–251.
[26] M. Esquenet, J.V. Swinnen, W. Heyns, G. Verhoeven, 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 (1996) 28.
[27] T.Y. Hsieh, C.Y. Ng, C. Mallouh, H. Tazaki, J.M. Wu, 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 (1996) 141–146.
[28] X.Y. Zhao, L.H. Ly, D.M. Peehl, D. Feldman, 1Alpha,25-dihydroxyvitamin D3 actions in LNCaP human prostate cancer cells are androgen-dependent, Endocrinology 138 (1997) 3290–3298.
[29] C. Gross, R.J. Skowronski, S.R. Plymate, J.S. Rhim, D.M. Peehl, D. Feldman, Simian virus 40-, but not human papillomavirus-, transformation of prostatic epithelial cells results in loss of growth-inhibition by 1,25-dihydroxyvitamin D3, Int. J. Oncol. 8 (1996) 41–47.
[30] C.M. Hansen, L. Rohde, M.W. Madsen, D. Hansen, K.W. Colston, G. Pirianov, P.K. Holm, L. Binderup, MCF-7/VD(R): a new Vitamin D resistant cell line, J. Cell Biochem. 82 (2001) 422–436.
[31] N. Akutsu, R. Lin, Y. Bastien, A. Bestawros, D.J. Enepekides, M.J. Black, J.H. White, Regulation of gene expression by 1alpha,25-dihydroxyvitamin D3 and its analog EB1089 under growth-inhibitory conditions in squamous carcinoma cells, Mol. Endocrinol. 15 (2001) 1127–1139.
[32] H.G. Palmer, M. Sanchez-Carbayo, P. Ordonez-Moran, M.J. Larriba, C. Cordon-Cardo, A. Munoz, Genetic signatures of differentiation induced by 1alpha,25-dihydroxyvitamin D3 in human colon cancer cells, Cancer Res. 63 (2003) 7799–7806.
[33] M. Camps, A. Nichols, S. Arkinstall, Dual specificity phosphatases: a gene family for control of MAP kinase function, FASEB J. 14 (2000) 6–16.
[34] T. Tanoue, T. Moriguchi, E. Nishida, Molecular cloning and characterization of a novel dual specificity phosphatase, MKP-5, J. Biol. Chem. 274 (1999) 19949–19956.
[35] A. Ravid, E. Rubinstein, A. Gamady, C. Rotem, U.A. Liberman, R. Koren, Vitamin D inhibits the activation of stress-activated protein kinases by physiological and environmental stresses in keratinocytes, J. Endocrinol. 173 (2002) 525–532.
[36] P. De Haes, M. Garmyn, H. Degreef, K. Vantieghem, R. Bouillon, S. Segaert, 1,25-Dihydroxyvitamin D3 inhibits ultraviolet B-induced apoptosis, Jun kinase activation, and interleukin-6 production in primary human keratinocytes, J. Cell Biochem. 89 (2003) 663–673.
[37] Y. Ji, G.P. Studzinski, Retinoblastoma protein and CCAAT/enhancer-binding protein beta are required for 1,25-dihydroxyvitamin D3-induced monocytic differentiation of HL60 cells, Cancer Res. 64 (2004) 370–377.
[38] S. Swami, N. Raghavachari, U.R. Muller, Y.P. Bao, D. Feldman, Vitamin D growth inhibition of breast cancer cells: gene expression patterns assessed by cDNA microarray, Breast Cancer Res. Treat. 80 (2003) 49–62.

[39] D.T. Lincoln, E.M. Ali Emadi, K.F. Tonissen, F.M. Clarke, The thioredoxin–thioredoxin reductase system: over-expression in human cancer, Anticancer Res. 23 (2003) 2425–2433.
[40] D. Mustacich, G. Powis, Thioredoxin reductase, Biochem. J. 346 (Pt 1) (2000) 1–8.
[41] R. Koren, I. Hadari-Naor, E. Zuck, C. Rotem, U.A. Liberman, A. Ravid, Vitamin D is a prooxidant in breast cancer cells, Cancer Res. 61 (2001) 1439–1444.
[42] A. Ravid, D. Rocker, A. Machlenkin, C. Rotem, A. Hochman, G. Kessler-Icekson, U.A. Liberman, R. Koren, 1,25-Dihydroxyvitamin D3 enhances the susceptibility of breast cancer cells to doxorubicin-induced oxidative damage, Cancer Res. 59 (1999) 862–867.
[43] E. Garcion, L. Sindji, G. Leblondel, P. Brachet, F. Darcy, 1,25-dihydroxyvitamin D3 regulates the synthesis of gamma-glutamyl transpeptidase and glutathione levels in rat primary astrocytes, J. Neurochem. 73 (1999) 859–866.
[44] P. Autzen, C.N. Robson, A. Bjartell, A.J. Malcolm, M.I. Johnson, D.E. Neal, F.C. Hamdy, Bone morphogenetic protein 6 in skeletal metastases from prostate cancer and other common human malignancies, Br. J. Cancer 78 (1998) 1219–1223.
[45] F.C. Hamdy, P. Autzen, M.C. Robinson, C.H. Horne, D.E. Neal, C.N. Robson, Immunolocalization and messenger RNA expression of bone morphogenetic protein-6 in human benign and malignant prostatic tissue, Cancer Res. 57 (1997) 4427–4431.
[46] S.S. Hannah, A.W. Norman, 1 alpha,25$(OH)_2$ Vitamin D3-regulated expression of the eukaryotic genome, Nutr. Rev. 52 (1994) 376–382.
[47] C. Carlberg, P. Polly, Gene regulation by Vitamin D3, Crit. Rev. Eukaryot. Gene. Expr. 8 (1998) 19–42.
[48] C.M. Hansen, K.J. Hamberg, E. Binderup, L. Binderup, Seocalcitol (EB 1089): a Vitamin D analogue of anti-cancer potential. Background, design, synthesis, pre-clinical and clinical evaluation, Curr. Pharm. Des. 6 (2000) 803–828.
[49] F. Yeung, W.K. Law, C.H. Yeh, J.J. Westendorf, Y. Zhang, R. Wang, C. Kao, L.W. Chung, Regulation of human osteocalcin promoter in hormone-independent human prostate cancer cells, J. Biol. Chem. 277 (2002) 2468–2476.
[50] B.J. Boyle, X.Y. Zhao, P. Cohen, D. Feldman, 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 (2001) 1319–1324.
[51] M.J. Campbell, G.S. Reddy, H.P. Koeffler, Vitamin D3 analogs and their 24-oxo metabolites equally inhibit clonal proliferation of a variety of cancer cells but have differing molecular effects, J. Cell Biochem. 66 (1997) 413–425.
[52] T. Nickerson, H. Huynh, Vitamin D analogue EB1089-induced prostate regression is associated with increased gene expression of insulin-like growth factor binding proteins, J. Endocrinol. 160 (1999) 223–229.
[53] Q. Wang, W. Yang, M.S. Uytingco, S. Christakos, R. Wieder, 1,25-Dihydroxyvitamin D3 and all-*trans*-retinoic acid sensitize breast cancer cells to chemotherapy-induced cell death, Cancer Res. 60 (2000) 2040–2048.
[54] B.D. Mahon, A. Wittke, V. Weaver, M.T. Cantorna, The targets of Vitamin D depend on the differentiation and activation status of CD4 positive T cells, J. Cell Biochem. 89 (2003) 922–932.
[55] M.L. Katayama, F.S. Pasini, M.A. Folgueira, I.M. Snitcovsky, M.M. Brentani, Molecular targets of 1,25(OH)2D3 in HC11 normal mouse mammary cell line, J. Steroid Biochem. Mol. Biol. 84 (2003) 57–69.
[56] J.B. Nauert, T.M. Klauck, L.K. Langeberg, J.D. Scott, Gravin, an autoantigen recognized by serum from myasthenia gravis patients, is a kinase scaffold protein, Curr. Biol. 7 (1997) 52–62.
[57] M.M. Facchinetti, A.R. de Boland, Effect of ageing on the expression of protein kinase C and its activation by 1,25$(OH)_2$-Vitamin D3 in rat skeletal muscle, Cell Signal. 11 (1999) 39–44.
[58] Z. Schwartz, V.L. Sylvia, D. Larsson, I. Nemere, D. Casasola, D.D. Dean, B.D. Boyan, 1Alpha,25(OH)2D3 regulates chondrocyte matrix vesicle protein kinase C (PKC) directly via G-protein-dependent mechanisms and indirectly via incorporation of PKC during matrix vesicle biogenesis, J. Biol. Chem. 277 (2002) 11828–11837.